Zinc as Activating Cation for Muscle Glycolysis

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Summary Zinc cation performed a role of activator as well as inhibitor for production of lactate from glucose 6-phosphate in the cytosol fraction of rat muscle. The pH optimum for glycolysis was 7.3 when Zn\(^{2+}\) acted as an activator. At concentrations lower than 0.46 mM, Zn\(^{2+}\) was shown to be a more effective activator than Mg\(^{2+}\) with an apparent \(K_{1/2}\) of approximately 0.1 mM. However, at concentrations higher than 0.5 mM, Zn\(^{2+}\) inhibited lactate production. The activatory as well as inhibitory effect of Zn\(^{2+}\) on lactate production was investigated by the estimation of glycolytic intermediates. From the crossover plot, lactate production reflected phosphofructokinase activity, when Zn\(^{2+}\) was used as a catalytic cation for both reactions. Phosphofructokinase activity in purified muscle was activated by Zn\(^{2+}\) with an apparent \(K_m\) of approximately 0.05 mM, but at high Zn\(^{2+}\) concentrations, the enzyme activity was inhibited with an \(I_{50}\) of 0.23 mM in the presence of ATP. From these findings, it appears that lactate production might also depend on phosphofructokinase activity when Zn\(^{2+}\) is used as an activating cation.

Key Words zinc, glycolysis, phosphofructokinase, glycolytic intermediates, lactate

The investigation of trace elements of dietary origin in animals has recently been emphasized. Zinc is an indispensable mineral element (1) and it has been proposed that metallothionein acts as the regulator of Zn\(^{2+}\) metabolism (2, 3). Zinc is an essential component of several enzymes, including dehydrogenase, in carbohydrate metabolism (4).

Quarterman (5) proposed that Zn\(^{2+}\) activated the incorporation of glucose into adipose tissue. Bargoni (6) reported that Zn\(^{2+}\) inhibited the formation of lactate by smooth muscle extracts. In a previous paper (7), we also proposed that Zn\(^{2+}\) strongly inhibited lactate production from glucose 6-phosphate in the cytosol.

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Abbreviations: G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate.
fraction of rat muscle and that the inhibitory effect on lactate production was completely reversed by the addition of histidine. The inhibitory site for Zn$^{2+}$ was found at a point between fructose 6-phosphate and fructose 1,6-bisphosphate. However, Zn$^{2+}$ could act as activating cation as well as inhibitor for fructose 1,6-bisphosphatase (8–10) and pyruvate kinase (11). The investigation of the effect of Zn$^{2+}$ on glycolysis should be important for resolving the physiological function of Zn$^{2+}$ and the mechanism of control of glycolysis under physiological conditions.

From these findings, we carried out more detailed studies on the effect of Zn$^{2+}$ on glycolysis. In the course of our studies we found that Zn$^{2+}$ could act as an activating cation for glycolysis and that purified phosphofructokinase was activated by Zn$^{2+}$.

**MATERIALS AND METHODS**

*Chemicals.* All chemicals used were of analytical grade and were purchased from Nakarai Chemicals Ltd., Kyoto, unless otherwise stated. All nucleotides and auxiliary enzymes were obtained from Boehringer Mannheim GmbH. Sephadex G-25 was purchased from Pharmacia.

*Animals.* Male Sprague-Dawley rats, weighing 200 to 250 g, were housed in individual screen-bottomed cages in a room maintained at 23±1°C with 50% humidity and light regulation (12 h light per day). The animals were fed on a commercial stock diet (Oriental Kobo Ltd.) and water ad libitum. After the sacrifice of the rats, gastrocnemius muscle was quickly removed to be provided as a sample for analysis of glycolysis and other skeletal muscle as material for phosphofructokinase preparation.

*Lactate production and estimation of glycolytic intermediates.* Sample preparation for lactate production and quantitative determination of glycolytic intermediates were followed as per a previous paper (7). A Sephadex G-25 column equilibrated with 20 mM potassium phosphate, pH 7.0, containing 280 mM KCl, 1 mM ATP and 1 mM dithiothreitol, was used to remove the low molecular weight substances from the cytosol fraction, and the protein fraction was used as the enzyme source. Glycolysis was measured as the rate of production of lactate from glucose 6-phosphate. The standard reaction mixture contained 40 mM triethanolamine–40 mM potassium phosphate, pH 7.4, 10 mM glucose 6-phosphate, 1.1 mM ATP, 28 mM KCl, 0.4 mM NAD, 0.1 mM dithiothreitol and 2.1 mg of the above enzyme protein in a final volume of 2.0 ml.

Protein concentration was measured by the biuret method with albumin as standard (12).

*Purification of phosphofructokinase and enzyme assay.* Rat skeletal muscle phosphofructokinase was isolated as described previously (7). The enzyme was dialyzed overnight at about 4°C against 50 mM potassium phosphate buffer, pH 7.1, containing 1 mM fructose 6-phosphate and 5 mM 2-mercaptoethanol before use and was diluted with the same buffer. The enzyme reaction was followed by measuring the rate of production of lactate from glucose 6-phosphate.
the rate of the disappearance of NADH at 340 nm, in a cuvette of 1.0 cm light path at 25°C and using a Hitachi spectrophotometer model 200-10. The standard reaction mixture contained 50 mM triethanolamine–50 mM diethanolamine buffer, pH 7.1, including 5 mM MgCl₂, 0.5 mM ATP, 3 mM fructose 6-phosphate, 0.25 mM NADH, 5 units triosephosphate isomerase, 5 units aldolase and 1 unit α-glycerophosphate dehydrogenase. The reaction was started by addition of phosphofructokinase. When Zn²⁺ functioned as the activating cation, the reaction was started by addition of Zn²⁺. One unit of phosphofructokinase was defined as the amount of enzyme which catalyzed the formation of 1.0 μmol of fructose 1,6-bisphosphate per min under the described conditions.

The enzyme was shown to be homogeneous by polyacrylamide gel electrophoresis in both the absence and the presence of SDS, and showed a specific activity of 140 μmol·min⁻¹·mg⁻¹ under standard assay conditions in the presence of 1 mM AMP at 25°C.

Quantitative determination of Zn²⁺ and Mg²⁺. Atomic absorption spectrometry was employed to measure the concentration of stock Zn²⁺ and Mg²⁺ solutions and that of the sample solutions, using a Hitachi 208 Atomic Absorption Spectrophotometer at 213.8 nm and 285.2 nm, respectively. Glassware used in preparation and storage of the samples was soaked in aqua regia and then rinsed well with doubly distilled deionized water.

RESULTS

Effect of Zn²⁺ on muscle glycolytic activity

In a previous paper (7), the cytosol fraction of muscle desalted low molecular weight substances with Sephadex G-25 activated lactate production in the presence of Mg²⁺. As Fig. 1 shows, zinc was able to replace Mg²⁺ as a catalytic cation for glycolysis. In the presence of 0.1 mM Zn²⁺, the lactate production was linear with

![Graph](image)

Fig. 1. Activation of muscle lactate production by Zn²⁺ or Mg²⁺. O, Zn²⁺; ●, Mg²⁺. These compounds were included in the standard assay mixture.

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time for 60 min at least and the pH optimum was about 7.3. However, several
divalent metal ions such as Ni²⁺, Cu²⁺, Co²⁺ and Ca²⁺ were not substituted at
0.1 mM.

In the absence of Mg²⁺, lactate production was activated by Zn²⁺ with an
apparent $K_m$ of 80 $\mu$M, less than the value of 200 $\mu$M estimated for Mg²⁺ (Fig. 1). At
concentrations lower than 400 $\mu$M, Zn²⁺ was found to be a more effective activator
than Mg²⁺. However, at concentrations higher than 500 $\mu$M Zn²⁺ lactate pro-
duction was inhibited, while Mg²⁺ increased such production at concentrations up
to 1 mM.

The concentrations of Zn²⁺ and Mg²⁺ at the enzyme source of glycolysis were
calculated to be 0.75 ± 0.03 nmol/mg protein ($n=5$) and 1.25 ± 0.11 nmol/mg pro-
tein ($n=5$), respectively. These endogeneous divalent cations may function in
glycolysis at a low velocity with no addition of divalent cation (Fig. 1).

The levels of glycolytic intermediates

Table 1 summarizes the results of experiments performed to determine for
Zn²⁺ the limiting site of glycolysis. When 10 $\mu$mol of glucose 6-phosphate were
added to the incubation mixture, the sum of glycolytic intermediates including

|          | Mg²⁺                  | Zn²⁺                  |
|----------|-----------------------|-----------------------|
|          | 0.2 mm (10⁻⁹ mol)     | 5.0 mm (10⁻⁹ mol)     |
| G-6-P    | 4,116 ± 377           | 2,281 ± 225           |
| F-6-P    | 2,819 ± 261           | 1,512 ± 51            |
| FBP      | 816 ± 106             | 964 ± 112             |
| DHAP     | 68 ± 12               | 117 ± 25              |
| GAP      | 51 ± 12               | 85 ± 21               |
| 1,3-BPG  | 146 ± 27              | 183 ± 33              |
| 3-PG     | 1,600 ± 289           | 2,091 ± 304           |
| 2-PG     | 299 ± 76              | 389 ± 66              |
| PEP      | 543 ± 94              | 671 ± 132             |
| Pyruvate | 31 ± 8                | 59 ± 10               |
| Lactate  | 1,315 ± 153           | 6,416 ± 439           |
| Total    | 11,804 ± 606          | 14,768 ± 1,005        |
|          | (9,818)               | (9,763)               |

|          | 0.2 mm (10⁻⁹ mol)     | 5.0 mm (10⁻⁹ mol)     |
| Zn²⁺     | 2,811 ± 390*          | 3,864 ± 347*          |
|          | 1,987 ± 232*          | 2,017 ± 220           |
|          | 965 ± 63              | 881 ± 74              |
|          | 98 ± 37               | 79 ± 21               |
|          | 64 ± 30               | 61 ± 29               |
|          | 171 ± 55              | 163 ± 47              |
|          | 1,914 ± 402           | 1,705 ± 381           |
|          | 388 ± 59              | 365 ± 88              |
|          | 644 ± 131             | 601 ± 92              |
|          | 40 ± 7                | 33 ± 8                |
|          | 4,671 ± 448**         | 2,819 ± 275**         |
|          | (9,758)               | (9,675)               |

*p < 0.05, ** p < 0.01 compared to isomolar Mg²⁺-groups.
glucose 6-phosphate was approximately 97%. The concentrations of glycolytic intermediates measured after incubation with equimolar amounts of Zn\(^{2+}\) were expressed as a percentage of the concentrations with 0.2 mM and 5.0 mM Mg\(^{2+}\), respectively, and are shown in the form of crossover plot (Fig. 2). At 0.2 mM of divalent cations, Zn\(^{2+}\) was more favorable for lactate production than Mg\(^{2+}\), but this was reversed at 5.0 mM. A crossover occurred at a point between fructose 6-phosphate and fructose 1,6-bisphosphate. The results indicated that the production of lactate might depend on phosphofructokinase activity.

**Effect of Zn\(^{2+}\) as a catalytic cation on phosphofructokinase activity**

In a previous paper (10), Zn\(^{2+}\) was found to strongly inhibit lactate production from glucose 6-phosphate in the cytosol fraction of rat muscle in the presence of 5 mM Mg\(^{2+}\). However, the above results suggest that Zn\(^{2+}\) may function as a catalytic cation for phosphofructokinase. In reality, Zn\(^{2+}\) acts as a catalytic cation for phosphofructokinase and, in the presence of ATP, the apparent \(K_m\) values were calculated to be 0.057 mM and 0.31 mM for Zn\(^{2+}\) and Mg\(^{2+}\), respectively (Fig. 3). However, the maximum activity obtained in the presence of Zn\(^{2+}\) was approximately 10\% that obtained with Mg\(^{2+}\). With GTP as phosphate donor, the enzyme was also activated by Zn\(^{2+}\) with a \(K_m\) of approximately 0.07 mM, which is less than the \(K_m\) of 0.12 mM estimated for Mg\(^{2+}\) (not shown in figure). The maximum velocity obtained in the presence of Zn\(^{2+}\) was 36\% that obtained with Mg\(^{2+}\), in the presence of GTP. At high concentrations of Zn\(^{2+}\), the enzyme activity was inhibited with an \(I_{50}\) of 0.23 mM in the presence of ATP.

The enzyme activity in the presence of Zn\(^{2+}\) showed sigmoid kinetics as regards the concentration of fructose 6-phosphate, with a Hill coefficient of 1.6, in the presence of ATP (Fig. 4). When GTP was used as phosphate donor, a hyperbolic
Fig. 3. Activation of purified muscle phosphofructokinase by Zn$^{2+}$ or Mg$^{2+}$. ●, Zn$^{2+}$; ○, Mg$^{2+}$. Other conditions were the same as the standard assay conditions.

Fig. 4. Phosphofructokinase activity as a function of fructose 6-phosphate by Zn$^{2+}$ or Mg$^{2+}$. ●, 0.25 mM Zn$^{2+}$; ○, 5 mM Mg$^{2+}$. Other conditions were the same as the standard assay conditions.

profile of phosphofructokinase activity as a function of fructose 6-phosphate concentration was shown. With Zn$^{2+}$ as the catalytic divalent cation, the velocity exhibits a hyperbolic dependence on the concentration of ATP with a $K_m$ of 1.32 mM, in the presence of 3 mM fructose 6-phosphate (not shown in figure).

**DISCUSSION**

In a previous paper (7), we described that Zn$^{2+}$ strongly inhibited production of lactate from glucose 6-phosphate in the cytosol fraction of rat muscle with an $I_{50}$
Table 2. Summary of the effects of Zn\textsuperscript{2+} on muscle glycolysis, and on phosphofructokinase, pyruvate kinase and fructose 1,6-bisphosphatase activities in the presence or absence of Mg\textsuperscript{2+}.

|                        | Mg\textsuperscript{2+} (mM) | Mode of action | $K_{1/2}$ ($\mu$m) | Reference |
|------------------------|-----------------------------|----------------|---------------------|-----------|
| Glycolysis             |                            |                |                     |           |
|                        | 5                           | inhibitor      | 10                  | (7)       |
|                        | 0                           | activator      | 100                 | this paper|
|                        | 0                           | inhibitor      | 3,000               | this paper|
| Phosphofructokinase    |                            |                |                     |           |
|                        | 5                           | inhibitor      | 10                  | (7)       |
|                        | 0                           | activator      | 57                  | this paper|
|                        | 0                           | inhibitor      | 230                 | this paper|
| Pyruvate kinase        |                            |                |                     |           |
|                        | 2.5                         | inhibitor      | 4.8                 | (11)      |
|                        | 0                           | activator      | 80                  | (11)      |
| Fructose 1,6-bisphosphatase |              |                |                     |           |
|                        | 5                           | inhibitor      | 0.5                 | (10)      |
|                        | 0                           | activator      | 10                  | (10)      |
|                        | 0                           | inhibitor      | 200                 | (10)      |

value of 10 $\mu$m. Tsuyuki and MacLeod (13) also proposed that Zn\textsuperscript{2+} also inhibited glycolysis in resting cell suspensions of L. arabinosus. However, Zn\textsuperscript{2+} also acted as a potent activator of lactate production in the absence of Mg\textsuperscript{2+} (Fig. 1 and Table 1). This would indicate that Zn\textsuperscript{2+} can act as activator as well as inhibitor of muscle glycolysis. As summarized in Table 2, Zn\textsuperscript{2+} was able to replace Mg\textsuperscript{2+} in glycolysis. Zinc, which acts as activator as well as inhibitor, was originally proposed as acting in this way on fructose 1,6-bisphosphatase activity by the Horecker group (8, 9). The mode of action of Zn\textsuperscript{2+} in muscle glycolysis resembled very much that with muscle fructose 1,6-bisphosphatase (10), phosphofructokinase (10) and pyruvate kinase (11). In the presence of Mg\textsuperscript{2+}, Zn\textsuperscript{2+} was a favorable inhibitor of the activity of these enzymes. However, in the absence of Mg\textsuperscript{2+}, Zn\textsuperscript{2+} could act as an activating cation at low concentrations and as an inhibiting one at high concentrations.

At 0.2 mM of a divalent cation, Zn\textsuperscript{2+} was more favorable than Mg\textsuperscript{2+} for muscle glycolysis (Table 1). The crossover data suggest that phosphofructokinase activity reflects the production of lactate and that Zn\textsuperscript{2+} acts as an activating cation for phosphofructokinase. In reality, phosphofructokinase was activated by Zn\textsuperscript{2+}, but not to a greater extent than with Mg\textsuperscript{2+} used as an activating cation, at all concentrations investigated (Fig. 3 and 4). Pontremoli et al. (14) proposed that the addition of aldolase reduced the binding of Zn\textsuperscript{2+} to the two high-affinity sites of
fructose 1,6-bisphosphatase. Therefore, in overall lactate production experiments, Zn$^{2+}$ bound to an inhibitory site of phosphofructokinase might be removed by proteins. The details are now under investigation.

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