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Studies on Myokinase in the Muscle of Fishes-III

Catalytic Properties of Myokinase I and II from Carp Muscle

Zentaro OOSHIRO*, Seiichi HAYASHI* and Masakazu FUKUSHIMA*

Abstract

Effect of bivalent metal ions on the activity of myokinase I and II from carp muscle was investigated. Co^{++}, Mn^{++} and Mg^{++} increased the activity of myokinase II by 160, 118 and 84 % respectively, but only Mg^{++} increased the activity of myokinase I slightly. EDTA inhibited the activity of myokinase II, but did not effect that of myokinase I. Myokinase II treated with EDTA was re-activated by dialyzing against Tris-Cl buffer (pH 7.6) containing $MgCl_2$ and $MnCl_2$. Mg^{++} increased the thermal stability of myokinase II. *Km* values for ADP of myokinase I and II were 13.3 mM and 6.7 mM at 20°C respectively. Activated energies of myokinase I and II were 6.75 and 5.61 Kcal per mole enzyme respectively.

Myokinase I and II were isolated and purified from carp muscle as described previously[1]. After gel filtration by Sephadex G-75, myokinase I and II were purified by DEAE-cellulose and CM-cellulose respectively. On the other hand it was reported by NODA *et al.*[2] that crystaline myokinase was isolated from carp muscle. They used phosphocellulose to purify the enzyme and used the buffer solution containing substrates, ATP and AMP, to elute the enzyme from phosphocellulose. However they reported there was no isozyme from carp muscle. Molecular weights of myokinase I and II were 27,000 and 22,000 respectively[1] and that of the enzyme isolated by NODA *et al.*[2] was 22,000.

We assumed that if myokinase I and II isolated by us were the artifact during the purification procedure, by such as proteases, catalytic properties of myokinase I and II were almost the same. If myokinase I and II were not the artifact, they had different catalytic properties. So we investigated the effect of bivalent metal ions and EDTA on the activity of the enzymes, thermal stability and kinetics of the enzymes. Optimal temperature, optimal pH and nucleotide specificity of the enzymes were reported previously[3]. Myokinase I and II showed the same optimal temperature, 35°C, the same optimal pH, pH 7.5, and the same nucleotide specificity.

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Experimental procedure

Preparation of myokinase I and II Myokinase I and II were prepared as described previously[1]. Carp muscle was homogenized with 5 volumes of 0.5% KCl. After salting out by $(NH_4)_2SO_4$, myokinase I and II were separated by gel filtration using Sephadex G–75. Furthermore myokinase I was purified by DEAE-cellulose and myokinase II was purified by CM-cellulose. Homogeneity of myokinase I and II was recognized by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Enzyme assay Myokinase activity was assayed by measurement of the amount of ATP produced by the enzyme using Dowex 1 × 4 (Cl type, 100–200 mesh) as described previously[1]. Assay mixture was 2.5 mM ADP and 25 mM Tris–Cl buffer (pH 7.6), and incubated at 30°C. Reaction was stopped by 30% perchloric acid. After filtration (Toyo No. 2), filtrate was neutralized with 5 N KOH. One enzyme unit is defined as the production of 1 μmole of ATP per min at 30°C.

Materials Dowex 1 × 4 (100–200 mesh) and ADP were purchased from Dow Chemical Co. and Sigma Chemical Co. respectively. Other reagents were obtained from Wako Pure Chemical Co. and Nakarai Chemical LTD.

Results

Effect of bivalent metal ions Effects of bivalent metal ions on the activity of myokinase I and II were investigated. Assay mixture was 2.5 mM ADP, 25 mM Tris–Cl buffer (pH 7.6), 0.5 mM bivalent metal ion and purified myokinase, and incubated at 37°C for 10 min. Table 1 shows the result. Effect of bivalent metal ions on the activity of myokinase I was clearly different from that on myokinase II activity. Co^{++}, Mn^{++} and Mg^{++} increased the activity of myokinase II by 161, 118 and 84% respectively. On the other hand the activity of myokinase I was increased only by Mg^{++} slightly, other bivalent metal ions such as Ca^{++}, Mn^{++}, Co^{++} and Zn^{++} inhibited the enzyme activity slightly.

Table 1. Effect of bivalent metal ions on the activity of myokinase I and II. Assay mixture was 2.5 mM ADP, 25 mM Tris–Cl buffer (pH 7.6), 0.5 mM bivalent metal ions and myokinase I (0.018 mg–N/ml) or myokinase II (0.017 mg–N/ml). Assay mixture was incubated at 37°C for 10 min.

Metal ion	Myokinase I		Myokinase II	
	ATP (μmole)	%	ATP (μmole)	%
None	0.301	100	0.121	100
Mg^{++}	0.336	112	0.222	184
Ca^{++}	0.247	82	0.067	56
Mn^{++}	0.297	98	0.264	218
Co^{++}	0.267	89	0.315	261
Zn^{++}	0.258	86	0.167	129

Thermal stability Purified myokinase I and II were incubated with 0.05 M Tris-Cl buffer (pH 7.6) alone or with 0.05 M Tris-Cl buffer containing 1 mM of $MgCl_2$, $MnCl_2$ or $CoCl_2$ at 50°C for various periods. After the appropriate period of incubation, the portion of the enzyme was cooled in ice bath and its activity was assayed. Myokinase I and II were inactivated by incubation at 50°C for 1 h and 12 h respectively (Fig. 1.). Myokinase I, which was incubated with the buffer containing $MgCl_2$ at 50°C for 1 h, was maintained 70% of inital activity. It was assumed that Mg^{++} protected myokinase I as well as myokinase II from denaturation by incubation at 50°C. Protective effect of Co^{++} or Mn^{++}, which increased the activity of myokinase II remarkably, on the thermal stability of myokinase II was not obserbed. Contrary the effect of Mg^{++} on the thermal stability of myokinase II, Co^{++} accelerated denaturation by incubation at 50°C.

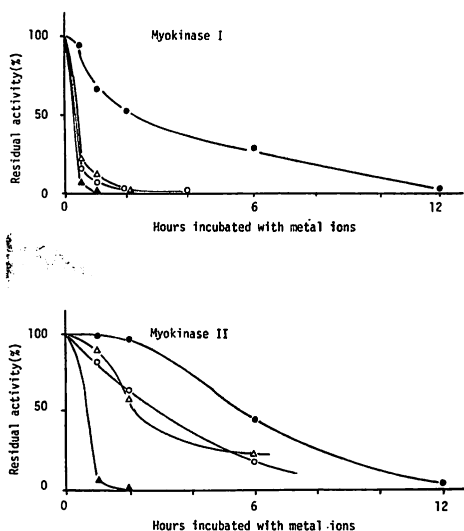

Fig. 1. Thermal stability of myokinase I and II.

After the appropriate period of incubation with or without bivalent metal ion at 50°C, the residual activity of the enzyme was assayed.

○——○ intact enzyme ●——● incubated with Mg^{++}
△——△ incubated with Mn^{++} ▲——▲ incubated with Co^{++}

Effect of EDTA Purified myokinase I and II were added in a equal volume of 0.05 M Tris-Cl buffer (pH 7.6) containing 0.1 M EDTA, and incubated in ice bath for 4 days. After the appropriate period of incubation, the portion of the enzyme was dialyzed against distilled water. Then the residual activity of the enzyme was assayed. As shown in Fig. 2., myokinase II was inhibited by EDTA almost completely. Contrary myokinase II, myokinase I was not inhibited by EDTA.

Re-activation of myokinase II treated with EDTA was investigated. Myoki-

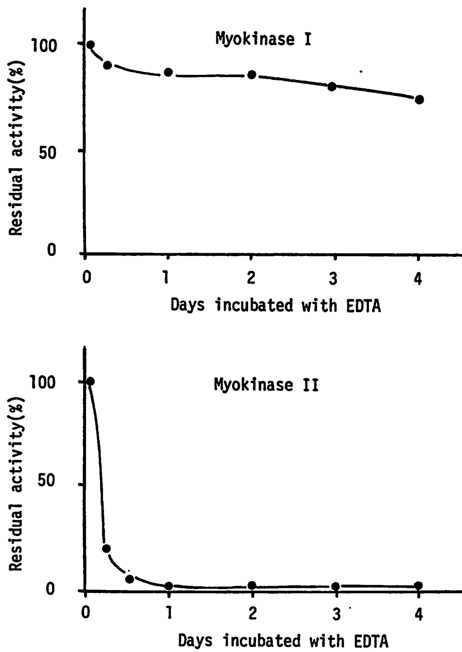

Fig. 2. Effect of EDTA on the activity of myokinase I and II.

After the appropriate period of incubation with 50 mM EDTA in ice bath, the enzyme was dialyzed and the residual activity was assayed.

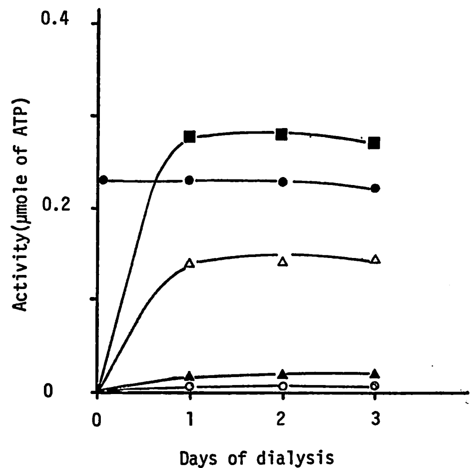

Fig. 3. Re-activation of myokinase II treated with EDTA.

After dialyzing the enzyme treated with 50 mM EDTA against 0.05 M Tris-Cl buffer (pH 7.6) containing 5 mM bivalent metal ion, the activity of the enzyme was assayed.

●——● intact enzyme
○——○ treated with EDTA and dialyzed against 0.05 M Tris-Cl buffer (pH 7.6)
■——■ treated with EDTA and dialyzed against 5 mM $MgCl_2$ solution
△——△ treated with EDTA and dialyzed against 5 mM $MnCl_2$ solution
▲——▲ treated with EDTA and dialyzed against 5 mM $CoCl_2$ solution

nase II treated with EDTA was dialyzed against 0.05 M Tris-Cl buffer (pH 7.6) containing 5 mM of $MgCl_2$, $MnCl_2$ or $CoCl_2$. Mg^{++} re-activated the enzyme treated with EDTA and the activity of re-activated enzyme showed almost equal to that of the intact enzyme (Fig. 3.). Mn^{++} also re-activated the enzyme treated with EDTA, but its activity was about 60% of that of intact enzyme. Co^{++} which most promoted the activity of myokinase II did not re-activate the enzyme treated with EDTA.

Kinetics of myokinase I and II The *Km* values for the substrate, ADP, of myokinase I and II were estimated by measuring ATP using Dowex 1× 4 resin descibed in "Experimental procedure". Fig. 4. shows Lineweaver-Burk plots for the substrate, ADP, at 20°, 30° and 40°C. In both cases linear reciprocal plots were obtained which allowed estimation of the *Km* values of myokinase I and II to be 17.40 and 7.37 mM at 30°C respectively. These values are somewhat higher than those reported for myokinases from the muscle of mammals[4], rat and bovine liver[4][5] and yeast[4].

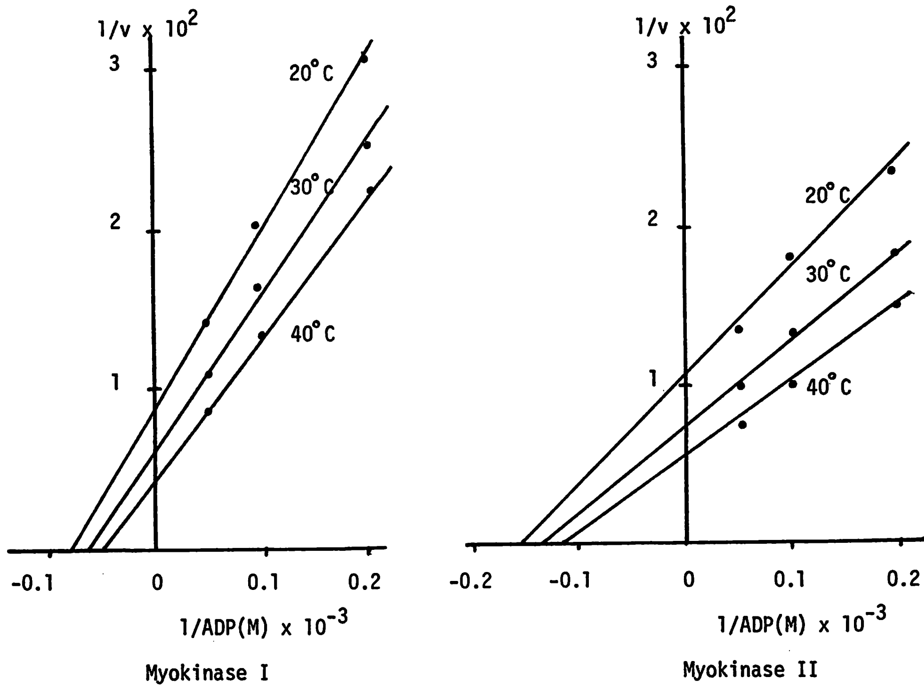

Fig. 4. Lineweaver-Burk plots for myokinase I and II.

The maximal velocities for the reaction, 2ADP→ATP+AMP, catalyzed by the carp muscle myokinase I and II were estimated by the intercepts on the velocity axis presented in Fig. 4. The values of myokinase I and II at 30°C and pH 7.6 were 171 and 139 moles of ATP formed per min per mole enzyme respectively. Molecular weight values of 27,000 for myokinase I and 22,000 for myokinase II were utilzed for these caluculations.

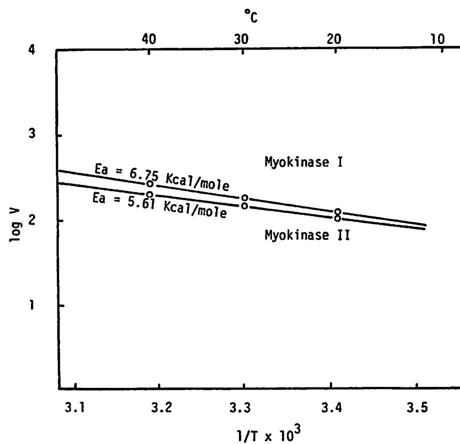

Fig. 5. Arrhenius plots for myokinase I and II.

Activated energies for myokinase I and II were obtained by Arrhenius plots and were 6.75 and 5.61 Kcal per mole enzyme respectively as shown in Fig. 5.

Discussion

We found that myokinase I and II which were recognized to be homogeneous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate had not only different molecular weight, but also different catalytic properties. Effect of bivalent metal ions and EDTA on the activity of the enzymes, thermal stability and kinetics of the enzymes were quite different from each other. Using ADP as substrate, the activity of myokinase II was increased by Co^{++}, Mn^{++} and Mg^{++}. However the activity of myokinase I was not so increased by Mg^{++} as that of myokinase II, and was inhibited by Mn^{++}, Ca^{++}, Co^{++} and Zn^{++}. If it is true as described by NODA[4] that the metal ion is combined with the nucleoside di- or triphosphate to form a complex, and that there are two substrate sites per enzyme active site - a site for binding a nucleoside monophosphate or a metal-free diphosphate and another site for binding metal-nucleoside triphosphate or a metal bound diphosphate, substrate sites of myokinase I and II seems to be different. It was also assumed from the effect of EDTA on the activity of myokinase I and II that the substrate sites of these enzymes were different. Myokinase I was not inhibited by EDTA, but myokinase II was inhibited extensively.

Myokinase II treated with EDTA was re-activated by dialyzing against Tris-Cl buffer containing $MgCl_2$. Mg^{++} increased the thermal stability of myokinase II as well as myokinase I.

From the results of kinetic studies, we found that either of myokinase I and II had higher values of *K*m for ADP than myokinases from other sources had. Further studies on kinetics under the presence of Mg^{++} may be required, since myokinase I and II need Mg^{++} for optimal activity.

We concluded that myokinase I and II were not the artifact during the purification procedure, by such as proteases. Because if they were the artifact, it was assumed that they had almost the same catalytic properties. However they had quite different catalytic properties. The discrepancy between the results on myokinases reported by us and by NODA *et al.*[2] is still remained. The recognition of myokinase I and II purified by the method of NODA *et al.*[2] will be necessary.

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