Properties of 5'-Nucleotidase from Carp Skeletal Muscle

Kazuko TOMIOKA*1 and Kinji ENDO*2

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The purified 5'-nucleotidase from carp muscle showed the optimum activity at about pH 7.5. At above this pH, Mg2+ affected the 5'-nucleotidase activity in a complex manner, showing a second optimum at pH 9 in the presence of some buffer ions. ADP and ATP were effective inhibitors of the enzyme in a manner of competitive inhibition. ADP was a far more powerful inhibitor than ATP. The enzyme activity was also inhibited by the addition of EDTA and the inhibition was dependent on the time of incubation with chelating agent. The inhibition of the enzyme by EDTA apparently followed a second order equation, log Vt/Vo = -k[EDTA]t. The apoenzyme which was prepared by treatment with EDTA restored the original activity effectively by the addition of Zn2+, and was more unstable on heating than the intact enzyme.

In the previous paper,1) the purification of 5'-nucleotidase (5'-nucleotide phosphohydrolase EC. 3.1.3.5) from skeletal muscle of carp has been described. It has also been shown that the purified 5'-nucleotidase was the key enzyme on the post-mortem degradation of inosine monophosphate (IMP) in carp muscle, and appeared to be membrane bound.

In order to control the post-mortem degradation of IMP in fish muscle during the storage, it would be useful to throw light on the enzymatic properties of 5'-nucleotidase. In the present work, some properties, mainly on the inhibitory effects of various compounds on carp muscle 5'-nucleotidase, are studied and discussed in comparison with 5'-nucleotidase from the other sources.

Materials and Methods

Reagents

Sephadex G-25 was purchased from Pharmacia Fine Chemicals, adenosine deaminase from Sigma Chemical Co. All other chemicals were reagent grade.

5'-Nucleotidase

The enzyme used in this experiment was prepared from the skeletal muscle of carp Cyprinus carpio as shown in the previous paper,1) and it was nearly homogenious on disc gel electrophoresis.

Assay of 5'-Nucleotidase

Two methods of assay were used for 5'-nucleotidase activity.

Assay 1 5'-Nucleotidase was assayed by the determination of the liberated orthophosphate as described previously.1) The reaction mixture contained 300 μmol of Tris-HCl buffer, pH 7.4, 0.5 μmol of AMP, 6 μg of adenosine deaminase, and 5'-nucleotidase in a final volume of 3.0 ml. The reaction, which was started by adding 5'-nucleotidase, was run at 20°C. The change in absorbance at 260 nm was followed spectrophotometrically with a Hitachi spectrophotometer model 220-S.

Results and Discussion

Effect of pH on 5'-Nucleotidase

The activity of the carp 5'-nucleotidase as a function of pH is shown in Fig. 1. The enzyme showed the optimum activity at a pH of about 7.5 in veronal-acetate-HCl buffer, and the activation of the enzyme with Mg2+ was observed at pH region greater than 8.0. In the pH region of 7.8 to 9.8, borate-NaOH buffer gave a lower activity than veronal-acetate-HCl buffer, but a similar activating effect of Mg2+ was observed in borate-NaOH buffer as in veronal-acetate-HCl buffer. In glycine-NaOH buffer, Mg2+ markedly activated the enzyme in the pH region examined, giving a
shoulder on the pH-activity curve at a pH of about 9. These results indicated that Mg\(^{2+}\) affected the 5'-nucleotidase activity at above optimum pH in a complex manner, showing a second optimum in the presence of some buffer ions at a pH of about 9.

Values of optimum pH of 5'-nucleotidase from various animal sources were in a range of 5.5-8.5, and those from cod muscle, rat head, and sheep brain were similar to the present value of carp 5'-nucleotidase. The effect of Mg\(^{2+}\) on the pH-activity curve observed here is consistent with the results obtained with 5'-nucleotidase preparations from cod muscle, carp muscle, and rat heart plasma membrane.

**Effect of Various Substances on 5'-Nucleotidase**

Relative activity of the carp 5'-nucleotidase in the presence of some of divalent metal ions and other substances is given in Table 1. The enzyme activity was markedly suppressed by Cu\(^{2+}\) and Zn\(^{2+}\), whereas Co\(^{2+}\) and Mn\(^{2+}\) were somewhat stimulatory. Little or no effect was observed by Mg\(^{2+}\) and Ca\(^{2+}\).

**Table 1. Effect of various substances on the activity of carp 5'-nucleotidase**

<table>
<thead>
<tr>
<th>Additives</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>1 mM</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>1 mM</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>1 mM</td>
</tr>
<tr>
<td>Co(^{2+})</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>0.01 mM</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>0.01 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>NaF</td>
<td>1 mM</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.01 mM</td>
</tr>
<tr>
<td>Inosine</td>
<td>1 mM</td>
</tr>
<tr>
<td>2'(3')-AMP</td>
<td>1 mM</td>
</tr>
<tr>
<td>5'-ADP</td>
<td>1 mM</td>
</tr>
<tr>
<td>5'-ATP</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

Relative activities were determined with additives range of 0.01-1 mM by using assay 1.

These results indicate that the addition of some divalent metal ions was not essential for the enzymatic activity. The enzyme was markedly inhibited by ethylenediaminetetraacetic acid (EDTA) and cysteine.

Of nucleotides examined, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) were also effective inhibitors of the enzyme.

**Kinetic Properties of 5'-Nucleotidase**

Kinetic properties of the carp 5'-nucleotidase were studied by employing different concentrations of IMP. A Michaelis constant of the 5'-nucleotidase for IMP was calculated to be 0.093 mM according to Lineweaver-Burk equation. This value is similar to that from carp muscle, bovine intestinal mucosa and rat heart sarcolemma, two orders of magnitude smaller than that of 5'-nucleotidase from pigeon heart, one order of magnitude smaller than that from cod muscle and bonito muscle, and one order of magnitude larger than that from rat heart and pig intestinal muscle.

The inhibitory effect of nucleoside di- and triphosphates on the hydrolysis of IMP by 5'-nucleotidase.
dase was investigated. As shown in Fig. 2, ADP and ATP depressed the dephosphorylation of IMP by 5'-nucleotidase in a manner of competitive inhibition. It was also found that ADP was a far more powerful inhibitor than ATP, Ki values of these compounds being 3.1 μM and 18 μM, respectively.

Fig. 2. Inhibitory effects of ADP and ATP on the activity of carp 5'-nucleotidase. Assay 1 was used except that IMP concentration was varied and other nucleotides were added.

The competitive inhibition by ADP and ATP has also been reported with several 5'-nucleotidases from other sources, 7, 10, 12, 13) and ADP was a more effective inhibitor than ATP in those enzymes. Therefore, the post-mortem degradation of IMP by 5'-nucleotidase in fish muscle must be depressed by ADP and ATP at some storage periods before the full rigor when the greater part of ADP and ATP in fish muscle disappears.

Inhibition of 5'-Nucleotidase by EDTA

Time course of inhibition of the carp 5'-nucleotidase in the presence of various levels of EDTA was investigated. The initial velocity of dephosphorylation of AMP by the enzyme was not affected by the addition of EDTA as shown in Fig. 3. The inhibition was dependent on the time of incubation with EDTA, and was markedly affected by the concentration of EDTA.

An inhibitory effect of EDTA has been reported on various 5'-nucleotidases from a wide variety of vertebrate sources. 5, 8, 9, 14) Although, in most cases, the inhibition by EDTA has been considered to be caused by chelating a bound metal ion in the enzyme, kinetics of the chelating have been remaining obscure. Then, on the assumption that the chelating would follow a second order equation, the logarithms of relative velocities of dephosphorylation of AMP, calculated from Fig. 3, were plotted against the reaction time. As shown in Fig. 4, the logarithms of relative velocities Vt/V0 were linearly decreased with the reaction time, depending upon the concentration of EDTA. The rate constant of the decrease in the velocity of the enzymatic activity in the presence of EDTA, or that of the inactivation of the 5'-nucleotidase by chelating a bound metal ion with EDTA, calculated from the slopes of lines in Fig. 4, gave 36.0 min⁻¹·M⁻¹ for 10⁻² M EDTA, 31.3 min⁻¹·M⁻¹ for 10⁻³ M EDTA, and 33.3 min⁻¹·M⁻¹ for 10⁻⁴ M EDTA. These values are substantially identical, and it is reasonable, therefore, to conclude that, in the presence of excess EDTA, the inactivation of the 5'-nucleotidase by chelating a bound metal ion with EDTA apparently follows a second order equation, log Vt/V0 = -k[EDTA]t, where [EDTA] is the concentration of EDTA added.

Effect of 5'-AMP on the Inhibition of 5'-Nucleotidase by EDTA

The rate constant of the inactivation of carp muscle 5'-nucleotidase in the presence of 10⁻³ M EDTA, decreased with increasing concentration
of AMP as shown in Fig. 5 and Fig. 6. This result indicates that there is a maximum limit of AMP concentration by which the rate constant is not affected. It is also suggested that the rate constant of the chelate reaction of EDTA with a bound metal ion in the intact enzyme is larger than that with a bound metal ion in the enzyme-substrate complex. Therefore, because of the decrease in the ratio of free enzyme to enzyme-substrate complex by increasing AMP concentration, the apparent decrease may occur in the rate constant of the inactivation of enzyme by EDTA. If so, it may be reasonable to suppose that the bound metal ion in 5'-nucleotidase may play a key role in the formation of an enzyme-substrate complex, and an affinity of the metal ion for the enzyme may be increased by the formation of an enzyme-substrate complex.

Reactivation of EDTA-treated 5'-Nucleotidase

The observations mentioned above raised questions of whether EDTA removes a bound metal ion in the carp 5'-nucleotidase or not, and whether the inhibitory effect of EDTA is reversible or not. An approach to the questions was then undertaken by studying with a apoenzyme which was prepared by incubating the intact enzyme with 1 mM EDTA at 30°C for 10 min, followed by gel filtration with Sephadex G-25. The activity of the apoenzyme was occasionally changed on the gel filtration with 0.01 M veronal-acetate-HCl buffer as an eluent. This is probably caused by traces of some metal.
ions from impurity of reagents or water used. Therefore, 0.01 M veronal-acetate-HCl buffer containing 1 μM EDTA was used for the gel filtration. The activity of the prepared apoenzyme did not fluctuate and the inhibitory effect of 1 μM EDTA on the activity was negligibly slight. The apoenzyme having 4.4% of the original activity was scarcely reactivated by the gel filtration. However, the apoenzyme was reactivated by the addition of divalent metal ions as shown in Fig. 7.

Of divalent metal ions examined, Zn$^{2+}$, Co$^{2+}$ and Mn$^{2+}$ reactivated the enzyme, and Zn$^{2+}$ was the most effective, occuring a maximum reactivation at 1.15 μM. The reactivation by Ni$^{2+}$, Cu$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$ was unsuccessful. The concentration of free divalent metal ions which was effective to restore the activity should be very low, because the apoenzyme solution contained 1 μM EDTA.

On the basis of the fact that the activity of the apoenzyme was fully restored by the addition of Zn$^{2+}$, it is reasonable to suppose that the time-dependent depression of dephosphorylation of AMP with 5'-nucleotidase in the presence of EDTA is caused by the reversible removement of the bound metal ion, which is essential for the enzymatic activity, especially for the formation of the enzyme-substrate complex.

With regard to the reactivation of 5'-nucleotidases, it has been shown that the particulate 5'-nucleotidase from bovine which was inactivated by acid and alkali treatments was partially reactivated with Zn$^{2+}$ and other divalent metal ions. On the other hand, it has been reported that the inactivation of 5'-nucleotidase from rat heart by EDTA was partially reversed by the removal of EDTA, and that the incubation of the EDTA-treated enzyme with various divalent metal ions failed to reactivate the enzyme.

Thermostability of EDTA-treated 5'-Nucleotidase

Potential enzymatic activity of the apoenzyme prepared as mentioned above was determined after the heat treatment at various temperatures. As shown in Fig. 8, the apoenzyme was unstable at temperature above 20°C, being much more labile on heating than the intact enzyme. This instability of the apoenzyme may indicate that the metal ion which was removed by EDTA should play a role in the stabilization of three-dimensional structure of the enzyme.

As mentioned above, the carp 5'-nucleotidase was found to be similar to 5'-nucleotidases from rat heart, pigeon heart, and pig intestinal muscle with respect to pH optima in the presence or absence of Mg$^{2+}$ and the inhibitory effect of ADP and ATP. This result suggests that the 5'-nucleotidase having the similar properties to carp enzyme is widely distributed in muscles of higher animals. Although it has been shown that the 5'-nucleotidase from heart muscle can act as a translo-
case of adenosine which is a powerful vasodilator,\textsuperscript{6) the physiological role of 5'-nucleotidase in skeletal muscle can not be well explained at present.

The inhibitory effect of EDTA on 5'-nucleotidase has been reported,\textsuperscript{5,8,9,14)} but the detailed mechanism of the inhibition has remained obscure. However, the present results indicate that EDTA may remove time-dependently a metal ion (perhaps Zn\textsuperscript{2+}) from the carp 5'-nucleotidase, and that the heat-labile and metal ion-free apoenzyme is instantaneously activated by the addition of a metal ion like Zn\textsuperscript{2+} which seems to be the most effective metal ion.

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References