Properties of ATP-Sulfurylase from Marine Alga
*Porphyra yezoensis*

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(Received February 16, 1988)

ATP-sulfurylase purified from *Porphyra yezoensis* showed maximal activity at pH 8.2, and 37°C. It was shown that the actual substrate was Mg\(^{2+}\)-ATP complex, and that ree-ATP was an inhibitor of the forward reaction. APS was a potent product inhibitor, competitive with respect to Mg\(^{2+}\)-ATP and a mixed type inhibitor with respect to molybdate. The K\(_m\) values for Mg\(^{2+}\)-ATP, molybdate, PPI, and APS were 0.67 mM, 6.7 mM, 33 \(\mu M\), and <1 \(\mu M\), respectively. Initial velocity studies showed that the mechanism of the molybdolysis reaction was a sequential type. The molybdolysis reaction was inhibited by several nucleotides and anions of a sulfate analog, but was not affected by p-chloromercuribenzoate.

ATP-sulfurylase (EC 2.7.7.4), the first sulfate activating enzyme which catalyzes the formation of APS*2 from sulfate and ATP, is widespread in nature.1,2) The yeast,3) *Penicillium chrysogenum*,4-10) cabbage leaf,11) spinach leaf,12,13) and rat liver14) enzymes have been highly purified and characterized in detail. Although ATP-sulfurylase has also been demonstrated in marine algae, very little is known about its properties, apart from the work of Moller and Evans which is concerned with the properties of the partially purified enzyme from unicellular red alga *Rhodella maculata*.15)

In the previous papers,16,17) we reported the purification and the APS synthesizing activity of ATP-sulfurylase from the red alga *Porphyra yezoensis*. This paper deals with some properties of *Porphyra* ATP-sulfurylase.

**Materials and Methods**

**Chemicals**

APS and PAPS were purchased from Sigma Chemicals. APS was also synthesized by the method of Cherniak and Davidson.18) Other nucleotides, phosphoenolpyruvate, and inorganic pyrophosphatase (PPase) were from Boehringer Mannheim. The inorganic PPase was desalted by using a Sephadex G-25 column prior to use. The hexokinase and glucose-6-phosphate dehydrogenase were from Oriental Yeast Co., Ltd. Other chemicals used were of the highest purity obtainable from commercial sources.

**ATP-sulfurylase**

ATP-sulfurylase was purified from *P. yezoensis*, collected at Sanriku, Iwate, Japan, in the manner described previously.16) The purified enzyme was homogeneous with respect to polyacrylamide gel electrophoresis, and the specific activity was 13.8 units/mg protein in the standard molybdolysis assay.16)

**Enzyme Assays**

ATP-sulfurylase activity was determined by the molybdolysis assay. The standard conditions of assay were described previously.16) The enzyme was assayed in the reverse direction (ATP synthesis) by the spectrophotometric method of Tweedie and Segel.19) The standard reaction mixture contained 1.0 \(\mu mol\) APS, 2.0 \(\mu mol\) Na\(^{+}\)-PPI, 5.0 \(\mu mol\) MgCl\(_2\), 0.6 \(\mu mol\) NADP, 1.0 \(\mu mol\) glucose, 2.5 units of glucose-6-phosphate dehydrogenase, 5.0 units of hexokinase, ATP-sulfurylase, and 100 \(\mu mol\) Tris-HCl (pH 8.2) in a final volume of 1 ml. The rate of NADP reduction, depending on the generation of ATP from APS and PPI, was monitored at 340 nm by a spectrophotometer with a thermostated cell holder (30°C). In some experiments, identification and quantification of the products (APS, AMP, and ATP) were carried out by anion-exchange HPLC on a Hitachi Gel #3013-N column.20) The enzyme activity is re-
ported in terms of micromoles of the product (PPi, AMP, or ATP) formed/min per mg protein.

Protein was determined by the method of Lowry et al. with bovine serum albumin as a standard.

**Results and Discussion**

**Effects of pH and Temperature**

ATP-sulfurylase from *Porphyra* showed the relatively broad activity optimum within the range of pH of 7.5–8.8, with a maximal activity at pH 8.2, in agreement with the results obtained with the enzymes from other organisms. The optimal temperature of the *Porphyra* enzyme was 37°C. This value is very low compared to the reports on the enzymes from other organisms, except the enzyme from *Rhodella*. The low temperature optimum of *Porphyra* enzyme seems to be associated with the fact that the laver grows in the winter. The activation energy was 9.5 kcal/mol (39.7 kJ/mol).

**Effect of Metal Ions**

The effect of metal ions (at 10 mM) on the ATP-sulfurylase activity was examined using a reaction mixture without inorganic PPase to eliminate the effect of metal ions on the activity of inorganic PPase. Prior to this, it was confirmed that the omission of inorganic PPase did not affect the molybdolysis reaction. The enzyme activity was determined by measuring a generated AMP on HPLC. The enzyme exhibited no activity without divalent cation. The highest activity was obtained with Mg²⁺. 86.33% and 8.5% of the activity were observed with Mn²⁺ and Co²⁺ respectively, while Ca²⁺, Cu²⁺, Fe²⁺, and Zn²⁺ did not replace Mg²⁺.

**Role of Mg²⁺**

Fig. 1-A shows that maximal activity is obtained in the presence of equal concentrations of Mg²⁺ and ATP, and that the presence of excess ATP to Mg²⁺ inhibited the activity. In contrast, excess Mg²⁺ had no effect on the activity. From these results, the real substrate of the enzyme was expected as a 1:1 complex of Mg²⁺ and ATP. Free-ATP was an inhibitor competitive with Mg²⁺-ATP (K_i=2.6 mM) as judged by a double reciprocal plot (Fig. 1-B) and Dexion plot of the data from Fig. 1-A. Our result agrees with the conclusion of Tweedie and Segel, and Osslund et al.

**Substrate Specificity**

Group VI anions, SO₄²⁻, SeO₄²⁻, CrO₄²⁻, WO₄²⁻, and MoO₄²⁻, reacted with Mg²⁺-ATP and released PPi in the presence of the enzyme. However, the examination of the reaction product on HPLC showed that sulfate was the only substrate accompanying the production of a stable adenyl anion. As summarized in Table 1, anions other than group VI were not effective as the substrate, but inhibited the molybdolysis reaction. Among them, chlorate which was
Table 1. Inhibition of the molybdolysis reaction\(^*1\) by nucleotides and anions

<table>
<thead>
<tr>
<th>Compound(^*2)</th>
<th>Inhibition (%)</th>
<th>Compound(^*2)</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>Adenine 25.3</td>
<td>HCOO(^-) 11.8</td>
<td>Adenosine 28.5</td>
<td>KI,(_{app.}) 0.6 mM</td>
</tr>
<tr>
<td>5'-AMP 50.5</td>
<td>NO(_2)(^-) 14.1</td>
<td>5'-ADP 50.5</td>
<td>S(_2)O(_3)(^-) 64.5</td>
</tr>
<tr>
<td>3'-AMP 33.5</td>
<td>NO(_3)(^-) 75.0</td>
<td>5'-CTP 38.8</td>
<td>ClO(_2)(^-) 91.8</td>
</tr>
<tr>
<td>5'-GTP 49.7</td>
<td></td>
<td>5'-ITP 32.3</td>
<td></td>
</tr>
<tr>
<td>5'-UTP 32.5</td>
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\(^*1\) 8 mM ATP, 15 mM MgCl\(_2\), 8 mM Na\(_2\)MoO\(_4\), 0.1 mM Tris-HCl (pH 8.2), excess inorganic pyrophosphatase, and ATP-sulfurylase in a total volume of 0.5 ml for 10 min at 30°C.

\(^*2\) Nucleotides were added at 4 mM, and anions were added at 5 mM (final conc.).

Fig. 2. Inhibition of the molybdolysis reaction by ClO\(_3\)\(^-\). A, reciprocal of velocity versus 1/[Mg\(^{2+}\)-ATP] at the fixed [MoO\(_4\)\(^{2-}\)] (10 mM) and the following [ClO\(_3\)\(^-\)]: (○), 0.0 mM; (□), 0.5 mM; (▲), 1.0 mM; (■), 2.0 mM; (●), 3.0 mM. The inset shows the replot of intercept versus [ClO\(_3\)\(^-\)]. B, reciprocal of velocity versus 1/[MoO\(_4\)\(^{2-}\)] at the fixed [Mg\(^{2+}\)-ATP] (10 mM) and the following [ClO\(_3\)\(^-\)]: (○), 0.0 mM; (□), 0.1 mM; (▲), 0.3 mM; (■), 0.5 mM; (●), 0.9 mM. The inset shows the replot of slope versus [ClO\(_3\)\(^-\)].

Fig. 3. Inhibition of the molybdolysis reaction by APS. A, reciprocal of velocity versus 1/[Mg\(^{2+}\)-ATP] at the fixed [MoO\(_4\)\(^{2-}\)] (10 mM) and the following [APS]: (○), 0.0 μM; (□), 1.0 μM; (▲), 2.0 μM; (■), 3.0 μM; (●), 5.0 μM. The inset shows the replot of intercept versus [APS]. B, reciprocal of velocity versus 1/[MoO\(_4\)\(^{2-}\)] at the fixed [Mg\(^{2+}\)-ATP] (10 mM) and the following [APS]: (○), 0.0 μM; (□), 2.0 μM; (▲), 4.0 μM; (■), 6.0 μM; (●), 10.0 μM. The inset shows the replot of intercept versus [APS].

The enzyme showed an absolute requirement for Mg\(^{2+}\)-ATP. CTP, GTP, ITP, and UTP were not active even in the presence of excess Mg\(^{2+}\). These nucleoside triphosphates as well as several adenine nucleotides inhibited the molybdolysis reaction (Table 1). It is concluded that the Porphyra enzyme has a high degree of catalytic

known as the dead-end inhibitor of ATP-sulfurylase\(^6,11\) showed a most prominent effect on the reaction of the Porphyra enzyme. The initial velocity study indicates that chlorate was competitive with molybdate (K\(_{i,app.}\) =0.6 mM) and uncompetitive with respect to Mg\(^{2+}\)-ATP (K\(_{i,app.}\) =1.0 mM) (Fig. 2).
specificity and a lower degree of binding specificity, like the *Penicillium* enzyme.10)

**Inhibition by APS**

It is noteworthy that the reaction with sulfate as the substrate was not linear as a function of the reaction time, or of the amount of enzyme presented, while the molybdolysis reaction showed good linearity. This finding suggests that the ATP-sulfurylase reaction is inhibited by the product, APS (in both cases, PPi is rapidly converted to PI). In the molybdolysis reaction, APS showed remarkable inhibition at micromolar concentrations. With the double reciprocal plot (Fig. 3) and the Dexion plot, it was determined that APS was a competitive inhibitor with respect to Mg$^{2+}$-ATP ($K_{i,app.}=1.0 \mu M$) and a linear mixed type inhibitor with respect to molybdate ($K_{i,app.}=8.0 \mu M$). This initial velocity pattern indicates that APS was a simple product inhibitor. Similar results have been reported for the enzyme from *Penicillium*5) and from cabbage leaf,11) while APS has been reported to be an allosteric inhibitor of the enzyme from yeast.3) It is interesting that another activated sulfate, PAPS, had no considerable effect on the reaction at 0.1 mM.

**Determination of $K_m$ Values**

A series of initial velocity studies were performed to establish the $K_m$ values of the enzyme for Mg$^{2+}$-ATP and molybdate. The double reciprocal plots of the data obtained by the reaction with the increasing concentrations of substrate at several fixed concentrations of the alternate substrate showed that the $K_m$ for each substrate varies with the concentration of the alternate substrate (Fig. 4-A, B). The intersecting patterns of the reaction indicated that both substrates bind to the enzyme before any product is released, namely, it is a sequential (ordered or random) mechanism. The initial velocity patterns of the product inhibition by APS, and of the dead-end inhibition by chlorate are consistent with an ordered mechanism in which Mg$^{2+}$-ATP adds before molybdate.7,11) The kinetics of the PPi product inhibition may make the mechanism more clear. However, this could not be investigated due to the nature of the molybdolysis assay. More detailed kinetic properties have been studied on the enzymes from *Penicillium*,5-10) cabbage leaf,11) and rat liver.14) In each case, it has been concluded that substrates bind in an ordered manner. The double reciprocal replots of the vertical intercepts versus each corresponding fixed substrate concentration were linear (Fig. 4, insets). Similar replots with respect to slopes were also linear. The $K_m$ for each substrate at an infinite concentration of the alternate substrate were calculated.

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**Fig. 4.** A, reciprocal of velocity versus $1/[\text{MoO}_4^{2-}]$ at several fixed [Mg$^{2+}$-ATP]: (●), 10.0 mM; (○), 4.0 mM; (▲), 3.0 mM; (△), 2.0 mM; (■), 1.0 mM; (□), 0.5 mM. The inset shows the replot of intercept versus $1/[\text{MoO}_4^{2-}]$. B, reciprocal of velocity versus $1/[\text{MoO}_4^{2-}]$ at several fixed [MoO$_4^{2-}$]: (●), 15.0 mM; (○), 10.0 mM; (▲), 7.0 mM; (△), 5.0 mM; (■), 3.0 mM. The inset shows the replot of intercept versus $1/[\text{MoO}_4^{2-}]$. 

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to be 0.67 mM for Mg2+-ATP and 6.7 mM for molybdate. The calculated V\textsubscript{max} was 31.3 units/mg. The Km value of the Porphyra enzyme for molybdate is markedly higher than the values reported for the enzymes from yeast,\textsuperscript{3} Penicillium,\textsuperscript{5} and cabbage leaf.\textsuperscript{11}

The effects of increasing APS and P Pi concentration on the rate of ATP-production were investigated at a saturating concentration of the other substrate (Fig. 5). Double reciprocal plots of the data from the saturation curve for each substrate were linear. K\textsubscript{m} values were calculated to be 33.0 \muM for P Pi, and to be below 1.0 \muM for APS. The calculated V\textsubscript{max} was 80.0 units/mg. In the reverse reaction, APS exhibited no substrate inhibition even at high concentrations, supporting the conclusion that APS was a simple product inhibitor.

Effect of PCMB

PCMB (1.0 mM) did not affect the enzyme activity, indicating that Porphyra ATP-sulfurylase does not have essential thiol groups on the enzyme activity.

It is recognized that the molybdoysis reaction gives valuable information on the enzymic properties of ATP-sulfurylase. However, it is necessary to elucidate the kinetics of the APS synthesizing reaction using a real substrate, sulfate, for the evaluation of sulfate metabolism in marine algae.

Acknowledgment

The authors wish to express their thanks to Mr. M. Fujita, a student of Kitasato University, for his technical assistance in this study.

References