Mevalonic Acid Kinase in Euglena gracilis

Carol Z. Cooper and C. R. Benedict
Chemistry Department, Wayne State University, Detroit, Michigan

Received December 22, 1966.

Summary. The isolation and partial purification of mevalonic acid kinase from Euglena gracilis is described. The product of the reaction MVA-5-P has been characterized by paper chromatography. The apparent 
Km values for L-mevalonic acid, ATP, and Mg

2+ are \(3 \times 10^{-5} \text{ M}, 6 \times 10^{-2} \text{ M}, \) and \(9 \times 10^{-3} \text{ M}, \) respectively. A concentration of \(1 \times 10^{-3} \text{ M} \) \(p\)-hydroxymercurobenzoate completely inactivates the enzyme. A distribution study has shown that mevalonic acid kinase is present in most higher plants and the algae Euglena gracilis and Chlamydomonas. No enzymatic activity could be detected in several species of photosynthetic bacteria or blue-green algae.

Mevalonic acid kinase has been studied in yeast (18), liver (13) and higher plants (14) but there has been no work on the phosphorylation of mevalonic acid in photosynthetic micro-organisms. In this paper, we have examined the characteristics of mevalonic acid kinase in partially purified preparations of Euglena gracilis and have examined the activity of this enzyme in extracts of blue-green algae and photosynthetic bacteria.

Materials and Methods

Materials. Sodium ATP and GSH were obtained from Sigma Chemical Company. D.L-Mevalonic acid-2-14C (DBED salt)\(^4\) was purchased from New England Nuclear Corporation. D.L-Mevalonic acid-5-phosphate (tris cyclohexylammonium salt) was purchased from Mann Research Company. Cultures. Euglena gracilis, Anabaena variabilis, Anacystis nidulans, Phormidium luridum, Synechococcus cedrorum and Nostoc muscorum were purchased from the Algae Culture Collection, Indiana University, Bloomington, Indiana. Chlamydomonas was purchased from Carolina Biological Supply Company, Burlington, North Carolina. Rhodospirillum rubrum (strain 1) was obtained from Dr. Howard Gest and Chromatium D was obtained from Dr. Martin Gibils. Baker's yeast, pig liver, and tomatoes were purchased in local markets.

Growth of Photosynthetic Microorganisms. All algae were grown in 2-liter bottles with continuous flushing with 1% CO\(_2\) in air in an illuminated water bath at 30\(^\circ\). Euglena gracilis was grown on Hutner's media as described by Greenblat and Schiff (9). Chlamydomonas was grown in a high-salt media as described by Sueoka (17). All species of the blue-green algae were grown on a media described by Kratz and Meyers (12).

Rhodospirillum rubrum was grown on malate and glutamate carbon sources as described by Kohmiller and Gest (11). The cells were grown anaerobically in glass stoppered bottles at 35\(^\circ\) in an illuminated water bath.

Chromatium D was grown on succinate as the sole carbon source as described by Hendley (10). The cells were grown anaerobically in glass stoppered bottles at 35\(^\circ\) in an illuminated water bath.

Enzyme Isolation. Four-day old cultures of Euglena gracilis were harvested by centrifugation and the cells washed 2 times with 0.1 M phosphate buffer pH 7.0. The cellular pellet was suspended in 25 to 50 ml of 0.1 M phosphate buffer pH 7.0 and \(1 \times 10^{-3} \text{ M} \) mercaptoethanol. The suspension was placed in a 10 Kc Raytheon oscillator for 10 minutes at three-fours power setting. The broken cell suspension was centrifuged in a refrigerated Sorvall centrifuge at 15,000 rpm for 20 minutes. The green supernatant fraction (crude fraction) was withdrawn and centrifuged in a Spinco model L centrifuge at 144,000 \(\times g\) for 60 minutes. The supernatant fraction was removed, the soluble protein precipitated with solid \((\text{NH}_4)_2\text{SO}_4\), and that protein which precipitated between 35 and 65% of saturation was collected by centrifugation. The protein precipitate was dissolved in 0.05 M phos-

---

\(^*\) This work was supported by Public Health Service grant GM-30428 from the National Institutes of Health.

\(^1\) Carol Z. Cooper is a recipient of a National Institutes of Health predoctoral fellowship GM 32 31401.

\(^2\) Present address: Agricultural Research Service, USDA, Department of Plant Sciences, Texas A & M University, College Station, Texas 77843.

\(^3\) The following abbreviations are used: MVA, mevalonic acid; DBED salt, N,N\(^1\)-dibenzylethylenediamine salt; MVA-5-P, mevalonic acid-5-phosphate; MVA-5-PP, mevalonic acid-5-phosphophate; and IPP, isopentenyl pyrophosphate.
buffer pH 7.0 and dialyzed for 1 and one-half hours at 4° against 7 liters of 0.005 M phosphate buffer pH 7.0 and 3 × 10⁻⁴ M mercaptoethanol. The dialysate was adsorbed onto a calcium phosphate gel, which had previously been batch equilibrated with 0.005 M phosphate buffer pH 7.0, at a gel:protein ratio of 3:1. The unadsorbed protein fraction was separated from the gel by centrifugation and used as the source of MVA kinase in the following experiments. The kinase in this fraction was purified 7 to 8 fold over the crude supernatant fraction.

Enzyme Isolation from Blue-Green Algae, Photosynthetic Bacteria, Liver, Yeast and Tomatoes. Enzyme isolation from the blue-green algae was essentially the same as for Euglena gracilis. The blue-green supernatant fraction isolated from the Sorvall centrifugation step was used as the source of mevalonic acid kinase.

The cells of the photosynthetic bacteria were collected by centrifugation and washed twice with 0.1 M phosphate buffer pH 7.0. The cellular pellet was suspended in 25 to 50 ml of 0.1 M phosphate buffer pH 7.0 and 1 × 10⁻³ M mercaptoethanol. The suspension was placed in a 10 Kc Raytheon oscillator for 90 seconds. The broken cell suspension was centrifuged in a Sorvall refrigerated centrifuge for 20 minutes at 15,000 rpm. The red supernatant fraction was used as the enzyme source.

A soluble supernatant fraction from dried baker's yeast was prepared by the procedure of Gailiusis et al. (8). A 35 to 65 % (NH₄)₂SO₄ fraction was prepared from pig liver soluble enzyme by the procedure of Benedict et al. (4). A soluble supernatant fraction was prepared from ripe red tomatoes by the procedure of Anderson and Porter (1).

Mevalonic Acid Kinase Assay. The reaction mixture contained in μmoles: 100, phosphate buffer pH 7.0; 10, ATP; 15, MgCl₂; 5, GSH; 10, NaF; 0.5, MVA (DBED salt) which contained 2 μc of MVA-2-¹⁴C, and 3 mg of protein in a total volume of 1 ml. The reaction mixture was incubated for 15 minutes at 37°. The reaction was stopped by adding 2 ml of boiling methanol and the reaction tube placed at 70° for 5 to 10 minutes. The precipitated protein was removed by centrifugation and the protein pellet washed twice with 1 or 2 ml of hot methanol. The supernatant fractions were combined and applied strip-wise to Whatman No. 3 filter paper. The radioactive compounds were separated by developing the papers in a solvent system of isopropanol: isoamyl alcohol: NH₄OH: H₂O (40:20:1:39 by volume). Non-radioactive mevalonic acid-5-phosphate was detected on the paper chromatograms at an Rₚ of 0.50 by spraying a section of the chromatograms with Hanes-Isherwood reagent for the detection of esterified phosphate compounds (2). The radioactive compounds in the reaction mixtures were located on the paper chromatograms by a Vanguard automatic chromagram scanner. The radioactive compound at Rₚ 0.50 was cut from the chromatograms and placed as a cylinder in a scintillation vial. Fifteen ml of scintillation fluor, composed of 9 g of 2,5-diphenyloxazole and 0.9 g of p-bis-2-(5-phenyloxazolyl)-benzene in 3 liters of toluene, was added to the vials and the samples assayed for radioactivity in a Packard liquid scintillation spectrometer.

Results

Chromatography. Figure 1 is a typical chromatogram scan of the reaction products resulting from the phosphorylation of MVA-2-¹⁴C in partially purified preparations of Euglena gracilis. The ¹⁴C-MVA-P produced in the reaction mixture and the authentic MVA-P have identical Rₚ values. We have shown that the ¹⁴C-MVA-P produced in these extracts of E. gracilis can be enzymatically converted to ¹⁴C-MVA-PP and ¹⁴C-IPP in extracts of pig liver.

![Chromatogram](https://example.com/chromatogram.png)

**Fig. 1.** Chromatographic separation of radioactive mevalonic acid phosphate and mevalonic acid from reaction mixtures of mevalonic acid kinase.

Localisation. The majority of the MVA kinase activity is found in the soluble supernatant fraction of the broken cell preparations (table I). No enzymatic activity could be found in the isolated chloroplast fraction. A large percentage of the protein in the crude fraction consisted of chloroplast protein. Since there is no kinase activity in the chloroplasts this may account for the lower amount of kinase in this fraction as compared to the soluble supernatant fraction. The MVA kinase was isolated and purified from the soluble supernatant fraction.

Table I. The Localization of Mevalonic Acid Kinase in Cellular Fractions of Euglena gracilis

<table>
<thead>
<tr>
<th>Cellular fraction</th>
<th>¹⁴C-MVA-P Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant</td>
<td>5083</td>
</tr>
<tr>
<td>Soluble supernatant</td>
<td>42,670</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>0</td>
</tr>
</tbody>
</table>
Table II. The Effect of Varying Cofactors on Mevalonic Acid Kinase Activity in Euglena gracilis

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>14C-MVA-P Formed (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>52,712</td>
</tr>
<tr>
<td>− ATP</td>
<td>2578</td>
</tr>
<tr>
<td>− MgCl₂</td>
<td>2421</td>
</tr>
<tr>
<td>− GSH</td>
<td>34,875</td>
</tr>
<tr>
<td>− NaF</td>
<td>62,962</td>
</tr>
<tr>
<td>+ Boiled enzyme</td>
<td>2406</td>
</tr>
</tbody>
</table>

Cofactors. Table II shows the effect on the MVA kinase activity caused by omitting different cofactors from the reaction mixture. Omitting ATP, MgCl₂ or substituting boiled enzyme, sharply reduces the formation of 14C-MVA-P. Withholding GSH from the reaction mixture reduces the kinase activity about 35%. Withholding NaF results in a slight increase in enzymatic activity; NaF is usually added to decrease the loss of ATP by phosphatase action. It is apparent that the MVA kinase in E. gracilis requires ATP and Mg²⁺ but does not show an absolute requirement for GSH. In this regard, MVA kinase from E. gracilis is inhibited 80% to 100% by 1 × 10⁻³ M p-hydroxymercuribenzoate.

Time and Protein. In separate experiments it has been shown that MVA kinase activity increases linearly with time to 40 minutes and increases linearly with protein concentrations to 3 mg. It is somewhat surprising that 3 mg of partially purified enzyme is required to convert 4% of 14C-MVA to 14C-MVA-P in 15 minutes. This amounts to the conversion of 2.6 μmoles of L-MVA to MVA-P per minute. This rate is comparable to the MVA kinase activity in pumpkin seedlings (14). These rates for E. gracilis and pumpkin seedling MVA kinase are well below the rates for the kinase from liver (13) and yeast (18). In liver and yeast experiments the kinase was assayed by coupling with purified pyruvate kinase.

Substrate. The effect of increasing the concentration of mevalonic acid on kinase activity is shown in figure 2. The specific activity of the radioactive mevalonic acid was constant in each of these reaction mixtures. These results show that the kinase is saturated at a concentration of MVA of 0.5 μmole per ml. The apparent Km for D-mevalonic acid is 6 × 10⁻⁴ M. The apparent Km for L-mevalonic acid is 3 × 10⁻⁸ M. The MVA kinase from E. gracilis exhibits a high affinity for MVA.

![Fig. 2. The effect of varying mevalonic acid concentrations on the mevalonic acid kinase activity in Euglena gracilis.](image)

ATP. The effect on MVA kinase activity of increasing the concentration of ATP is shown in figure 3. The enzyme is saturated with ATP at a concentration of 15 μmoles per ml. The apparent Km for ATP is 6 × 10⁻³ M. The enzyme is drastically inhibited at concentrations of ATP above 15 μmoles per ml. The inhibition at high ATP concentrations may be due to the fact that the ATP : MgCl₂ ratio greatly exceeds one.

MgCl₂. There is a noticeable lag in the production of mevalonic acid phosphate at low MgCl₂ concentrations (fig 4). Unlike the enzyme inhibition caused by high ATP concentrations, high MgCl₂
concentrations do not inhibit the enzyme even though the MgCl₂ : ATP ratio exceeds one. The apparent Km for MgCl₂ is $9 \times 10^{-3}$ M.

**Distribution.** The data in table III shows the distribution of MVA kinase in a variety of higher plants, yeast, liver and photosynthetic microorganisms *E. gracilis* and *Chlamydomonas*. Previously (15), an active preparation capable of converting $^{14}$C-MVA to MVA-P, MVA-PP or allyl pyrophosphates could not be obtained from tomato fruits. Our present work shows that MVA kinase is easily detectable in tomatoes. In this present study MVA kinase activity could not be detected in extracts of *R. rubrum*, *Chromatium* or several species of blue-green algae.

**Discussion.**

In this paper we have examined the characteristics of MVA kinase from extracts of *E. gracilis*. The cofactor requirements of this enzyme in *E. gracilis* do not differ substantially from the kinase in yeast, liver and higher plants (13, 14, 18). All of these enzymes are inhibited by $p$-hydroxymercuribenzoate and have similar Km values for ATP, Mg$^{2+}$ and mevalonic acid. The inhibition of the enzyme by high concentrations of ATP may be a distinguishing feature of MVA kinase from *E. gracilis*, but in most respects the enzyme resembles the kinase from higher plants.

It may be constructive to comment on MVA kinase activity in organisms in relation to their sterol and carotene content. MVA kinase is easily detectable in organisms or tissues like yeast or pig liver which are known to contain sterols (13, 18), and is similarly detectable in higher plants and algae which contain sterols and carotenoids (6,7, 16). However in this study, we have been unable to detect MVA kinase activity in blue-green algae or photosynthetic bacteria which do not contain sterols (3, 5) although they contain a variety of carotenoids. Thus while MVA kinase activity is high in organisms which contain sterols, in the blue-green algae and photosynthetic bacteria which contain carotenoids but lack sterols, the MVA kinase is either (a) absent, (b) below our present assay, or (c) substantially different from the kinase found in sterol synthesizing organisms.

**Literature Cited**


