Enzymatic Properties of Phosphogluconate Dehydrogenase in Rabbit Gingiva

by

Tadao FUJIWARA, Fumiyuki KUWATA, Masanori MATSUMURA, and Kantaro SUZUKI

Introduction

Phosphogluconate dehydrogenase (EC 1.1.1.44), a member of enzyme consisting of pentose phosphate pathway catalyzes the oxidative decarboxylation of 6-phosphogluconate, with NADP being reduced to NADPH, to yield ribulose 5-phosphate and CO₂. Many papers have been published on the enzymatic properties of phosphogluconate dehydrogenase in the liver of rat[1,2], sheep[3,4], and pig[5], and in bacteria[6-12]. On oral tissues, ROSETT et al.[13] and SIMPSON[14,15] assayed the activity levels of the enzyme in bovine and rat gingiva respectively. SIMPSON[14] has reported on the optimal pH and Mg²⁺ ion requirement of the enzyme activity. To investigate glucose metabolic pathway related to periodontal disease in human gingiva, it is necessary to be informed in detail about the enzymatic properties of each enzyme involved. In the present paper, phosphogluconate dehydrogenase from rabbit gingiva is partially purified by column chromatography on DEAE-cellulose, and the enzymatic properties are described.

Materials and Methods

Materials—Albino rabbits of both sexes, weighing about 2 kg, were used in this study. The head was stored frozen until use and employed within 5 days. Gingival fragments were obtained from the jaw, by pressing the safety razor against the attached gingiva of upper and lower jaws and then drawing it along the lingual or buccal side. For ease of homogenization the gingival fragments obtained was cut in slices with the razor on a glass-plate set on ice.

Enzyme preparations—All procedures were carried out at 4°C. The rabbit gingiva (wet weight: 500 mg) was homogenized in 5 ml of 5 mM TEA buffer, pH 7.8, with a glass homogenizer, and then centrifuged at 9,000 x g for 15 minutes. The supernatant fraction was applied on DEAE-cellulose column (1 x 20 cm) equilibrated with the above buffer. The elution was performed with a stepwise addition of NaCl at 0 M, 0.02 M, 0.08 M, 0.2 M and 0.5 M concentrations in the same buffer, to give 5 ml for each fraction.

Abbreviations used: DEAE-cellulose, O-(diethylaminoethyl)-cellulose; TEA, triethanolamine; PGA, 6-phosphogluconate; Tris, tris(hydroxymethyl)aminomethane; Fru-1,6-P₂, fructose 1,6-bisphosphate; PCMB, p-chloromercuribenzoate; PLP, pyridoxal 5'-phosphate.

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Enzyme assay—The reaction mixture was composed of TEA buffer 20 μmole, pH 7.8, PGA\(^c\) 0.6 μmole, NADP\(^d\) 0.08 μmole, MgCl\(_2\) 6.0 μmole and the enzyme preparation, in a final volume of 0.4 ml. The reaction was carried out for 30 minutes at 37°C, and terminated by the addition of 0.2 ml of 0.2 N NaOH. After the solution was heated for 15 minutes at 70°C, 3.4 ml of 8 N NaOH containing 0.01% hydrogen peroxide was added to be reincubated for 1 hour at 37°C. The fluorescence originating from the NADPH formed was measured with a fluorescence spectrophotometer\(^d\) at an excitation wavelength of 365 nm and an emission wavelength of 470 nm. The relationship between the enzyme concentrations and the amounts of NADPH formed was observed to be linear up to approximately 1.2 × 10\(^{-3}\) μmole of NADPH under the standard assay conditions as described above. One unit of enzyme activity was defined as the amount of enzyme required to convert 1.0 pmole of NADP to product for 1 minute at 37°C. All chemicals tested for their effects on enzyme activity were of the best commercially available grade, and were used without further purification. Each chemical was dissolved in 5 mM TEA buffer, pH 7.8, and the solution were adjusted to pH 7.8, before each experiment.

\[^c\] Boehringer Mannheim GmbH, West Germany.

\[^d\] Hitachi 204 fluorescence spectrophotometer, Hitachi, Japan.

Fig. 1. DEAE-cellulose column chromatography of phosphogluconate dehydrogenase. The elution of proteins from DEAE-cellulose column (void volume: 8 ml) was performed with a stepwise addition of NaCl at concentrations as indicated. The effluent fractions of 5 ml each were collected and assayed for the enzyme activity.
Protein determination—Protein concentration was determined with reagent kit* by the method of Bradford[16]. Bovine gamma globulin$^*$ was used as standard.

Molecular weight—The molecular weight was determined by the upward flow technique on a 2.6 × 100 cm column of Sephadex G-100$^t$, equilibrated with 50 mM TEA buffer, pH 7.8, containing 0.1 M KCl. The elution of proteins was carried out at 4°C with the same buffer and the fractions of 2.5 ml each were collected and assayed for the enzyme activity. The column was previously calibrated with the following proteins$^e$ of known molecular weight: aldolase (158,000); bovine serum albumin (67,000); chymotrypsinogen A (25,000); cytochrome C (13,500).

Results

Partial purification by chromatography on DEAE-cellulose—The elution profile of enzyme activity is illustrated in Fig. 1. The major enzyme activity was eluted as a single peak with 0.08 M NaCl in 5 mM TEA buffer, pH 7.8. The active fraction was tested for phosphogluconate dehydrogenase activity under the standard assay conditions excluding PGA or NADP, and for glucose 6-phosphate dehydrogenase ac-

![Fig. 2. Molecular weight determination of phosphogluconate dehydrogenase. Sephadex G-100 column was equilibrated with 50 mM TEA buffer, pH 7.8, containing 0.1 M KCl, prior to gel filtration. The column was calibrated with the standard proteins, which were monitored by absorbance at 280 nm. The effluent fractions of 2.5 ml each were collected and assayed for the enzyme activity.](image)

$^e$ Bio-Rad Laboratories, USA.

$^t$ Pharmacia Fine Chemicals, Sweden.
tivity under those containing glucose 6-phosphate as a substitute for PGA. In all cases, no NADPH formation could be detected. The active fraction served as the enzyme preparation in this study. The specific activity was estimated as 61 m units/mg protein, and about two-fold higher than that of the supernatants.

**Molecular weight**—Molecular weight was determined by gel filtration on Sephadex G-100. As shown in Fig. 2, the major enzyme activity was observed to be eluted at a position corresponding to a molecular weight of approximately 100,000.

**Effects of pH and buffers**—The pH of maximum enzyme activity was measured with the following buffers: 50 mM TEA; 50 mM Tris/HCl; 50 mM glycine/NaOH. The profile of maximum enzyme activity was observed to be nearly constant in the wide range of pH 7.4 to pH 9.4, as can be seen from Fig. 3. A pH of 7.8 in TEA buffer was used for the standard assay.

**Effects of divalent cations**—The effects of various divalent cations on enzyme activity were determined by adding various chloride salts to the reaction mixture under the standard assay conditions instead of Me ion (Table 1). In the presence of Mg²⁺ or Ca²⁺ ion, there was an increase in enzyme activity with higher concentrations of cation. Maximum activity was attained at a concentration of 10 mM. The maximum activity with Mg²⁺ ion was found to be slightly higher than that with Ca²⁺ ion. Mg²⁺ ion at a saturating concentration of 25 mM was used as the activating ion in the assay procedures. In contrast with this, other divalent cations tested showed inhibitory effects on enzyme activity. A significant difference was observed among those divalent cations, and Cd²⁺, Cu²⁺ and Zn²⁺ caused remarkable inhibitions.

![Fig. 3. Effects of pH and buffers on enzyme activity. Assays were performed as described under "Materials and Methods", except that pH and buffers were varied as indicated. O, 50 mM TEA buffer; ▲, 50 mM Tris/HCl buffer; ●, 50 mM glycine/NaOH buffer.](image)
**Table 1. Effects of divalent cations on enzyme activity.**

<table>
<thead>
<tr>
<th>divalent cations</th>
<th>enzyme activity* (munits) at divalent cation concentrations (mM)</th>
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<tbody>
<tr>
<td></td>
<td>0.1 mM</td>
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<tr>
<td>None</td>
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<tr>
<td>Mg²⁺</td>
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<tr>
<td>Ca²⁺</td>
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<td>Cu²⁺</td>
<td>0.01</td>
</tr>
<tr>
<td>Zn²⁺</td>
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* Instead of Mg²⁺ ion containing in the standard assay conditions as described under "Materials and Methods", each cation as indicated was added to the reaction mixture and then assayed for the enzyme activity.

Fig. 4. Heat stability of phosphogluconate dehydrogenase at 37°C. The preincubation mixture was composed of TEA buffer 20 μmole, pH 7.8, enzyme preparation and a chemical, in a volume of 0.25 ml. After the solutions were preincubated for the intervals indicated at 37°C, they were placed in an ice bucket and then assayed for the enzyme activity. ○, enzyme preparation; ●, enzyme preparation plus PGA 0.6 μmole; □, enzyme preparation plus NADP 0.08 μmole.

*Heat stability*—Figure 4 shows the time courses of the heat inactivation of enzyme, and of the protection of enzyme by substrates from the inactivation. As shown in Fig. 4, the enzyme activity was observed to retain 40% of its original activity after the preincubation for 30 minutes. However, addition of PGA completely protected the enzyme against the heat inactivation, whereas NADP resulted in slightly less protection. Furthermore, the possible protection of enzyme activity by a variety of amino acids was tested by adding to the preincubation mixture. All amino acids

- Amino acids tested: histidine; cysteine; valine; lysine; proline; alanine; threonine; serine; leucine; glycine; phenylalanine.
tested proved, more or less, to protect the enzyme against the heat inactivation. Especially, cysteine, threonine, leucine and glycine were most effective.

**Kinetic studies**—The kinetic pattern on the reaction mechanism was determined by changing a substrate concentration at various fixed levels of the other substrate. As shown in Fig. 5, parallel straight lines obtained indicated that the mechanism was consistent with a ping-pong mechanism as proposed by Cleland[18]. Km values for both PGA and NADP were derived as 0.05 mM from secondary plots of intercepts in Fig. 5.

Fig. 5. Kinetics of phosphogluconate dehydrogenase. The data are represented as double reciprocal plots of the initial velocities against PGA (in A) or NADP (in B), according to Line-Weaver and Burk[17]. In A, PGA was varied as indicated, at fixed NADP concentrations of 0.025 mM (●), 0.0375 mM (□), 0.05 mM (■), and 0.1 mM (○). In B, NADP was varied as indicated, at fixed PGA concentrations of 0.025 mM (●), 0.05 mM (□), 0.1 mM (■), and 0.15 mM (○).

<table>
<thead>
<tr>
<th>Table 2. Effects of metabolic intermediates on enzyme activity.</th>
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<tr>
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<td>glucose 6-phosphate</td>
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<tr>
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<tr>
<td>fructose 1-phosphate</td>
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</tr>
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Assays were performed in the presence of each metabolic intermediate concentration as indicated.
Effects of metabolic intermediates—Effects of metabolic intermediates on the catalytic function of enzyme were examined. As seen from Table 2, only Fru-1,6-P₂ caused a marked inhibition, whereas other intermediates had little effects on enzyme activity. Similar measurements demonstrated that 5 mM adenine nucleotides also exerted little influence on enzyme activity. In order to determine the inhibition type of Fru-1,6-P₂, the activity measurements as a function of substrate concentration were analyzed by means of double reciprocal plots. As a result, it was found to be competitive with respect to PGA and uncompetitive with respect to NADP (Fig. 6).

Inhibition of PCMB and restoration by sulfhydryl reducing reagents—By adding PCMB to the reaction mixture, the enzyme activity was found to be completely lost at a concentration of 5 μM. Kinetic studies on the inhibition type indicated it to be the same type as that of Fru-1,6-P₂. In order to detect the possible restoration from the inactivation caused by PCMB, the enzyme preparation was preincubated with PCMB 5 nmole and TEA buffer 20 μmole, pH 7.8, for 5 minutes at 37°C, and was then assayed by adding each sulfhydryl reducing reagent to the reaction mixture. The restoration was measured as the increase of enzyme activity. 5 mM cysteine gave the partial restoration, while 5 mM mercaptoethanol and 5 mM glutathione (reduced) had no effects.

Inhibition of PLP and restoration by amino acids—The enzyme activity was measured to be only 20% of its original activity by 1 mM PLP to the reaction mixture. The kinetic measurements on the inhibition type demonstrated that PLP inhibited competitively with PGA and uncompetitively with NADP. The restoration of enzyme activity from the inactivation by PLP was tested with a variety of amino acids. The inactivation due to the preincubation of enzyme preparation with PLP 0.2 μmole at 37°C was found to be restored completely by histidine, lysine and
phenylalanine, and partially by other amino acids except proline. In order to determine the restoration process, Km values with respect to PGA were evaluated on the enzyme restored partially in activity by lysine and valine. It may be easily found from Fig. 7 that the Km value of the enzyme restored partially by those amino acids was identical to that of the native enzyme measured under the standard assay conditions containing PLP 0.2 μmole.

**Discussion and Conclusions**

In studies reported here, phosphogluconate dehydrogenase from rabbit gingiva was partially purified with DEAE-cellulose column chromatography and characterized as to some enzymatic properties. In the procedures on chromatography, the enzyme was eluted as a single peak with 0.08 M NaCl. The specific activity was calculated as 61 m units/mg protein. The molecular weight of the enzyme was determined to be about 100,000, like that from rat liver[2]. SIMPSON[14] and deSHAZER[19] observed pH optima of pH 9.0 in rat gingiva and of pH 8.7-8.8 in bovine dental pulp, respectively. In rabbit gingiva, pH optimum was found to be in the wide range of pH 7.4 to pH 9.4, and to be discriminated from those in rat gingiva and bovine dental pulp. SIMPSON[14] reported that MgCl₂, in a final concentration of 0.1 M, caused 2.75-fold activation. On the other hand, TOEWS et al.[5] pointed out that pig liver phosphogluconate dehydrogenase did not require Mg²⁺ ion for activity. In the present measurements, addition of Mg²⁺ ion to the assay mixture brought about 1.4-fold activation, and the enzyme was activated by Ca²⁺ ion too, to a slightly lower extent. In contrast, Cd²⁺, Cu²⁺ and Zn²⁺ ions caused striking inhibitions in activity.
The effects of divalent cations thus obtained are similar to the findings by Glock and McLean[1].

The heat stability of enzyme activity was observed to be less stable, in common with that reported by Scott and Abramsky[8]. The heat inactivation was found to be protected, completely or partially, by PGA, NADP and many amino acids.

The results obtained in kinetic studies indicated the reaction mechanism as a ping-pong type. The ping-pong mechanism, presumed on the basis of the present kinetic measurements, is consistent well with the inhibition type of inhibitors as described in this paper. Hitherto, a random mechanism has been postulated in Candida utilis[9], and a sequential mechanism in sheep liver[3] or pig liver[5]. The reaction mechanism of rabbit gingiva phosphogluconate dehydrogenase appears to be possibly different from either mechanism as postulated with other sources. It was observed to be closely similar between Km value for PGA and that for NADP, and it was evaluated as 0.05 mM. These are of the same order as Km values given with other sources[2,6-8].

A variety of metabolic intermediates of the pathway relevant closely to the pentose phosphate pathway were investigated on the possible regulatory significance in metabolic control. Among chemicals tested in this experiment, only Fru-1,6-P2 was observed to exert a remarkable inhibitory effect. The kinetic measurements revealed the inhibition type to be competitive with respect to PGA and uncompetitive with respect to NADP. Ki value for PGA was calculated to be $8.6 \times 10^{-5}$ M, similar to that observed by Dyson and D’orazio[4]. The enzyme activity was confirmed not to be affected by adenine nucleotides, fructose 1-phosphate, fructose 6-phosphate or glucose 6-phosphate, even in most higher concentrations tested. Therefore, it seems improbable that the inhibition of Fru-1,6-P2 may be attributed to the similarity in molecular structure among such chemicals alone. It may be suggested that Fru-1,6-P2 level takes close part in the flow rate of the pentose phosphate pathway through the regulation of enzyme activity.

The inhibitory effect of PCMB indicated that a sulfhydryl group at the active center might participate in the catalytic reaction of enzyme. In contrast with mercaptoethanol and glutathione (reduced), cysteine was observed to be effective in the restoration of enzyme activity from the inactivation by PCMB. The results appear to be suggestive on the presence of cysteine residues at the active center, as previously indicated with other sources[1,11,12].

Finally, the inhibition of PLP was observed in the enzyme activity, and the inhibition type was found to be of the competitive type with respect to PGA and of the uncompetitive type with respect to NADP. The inactivation by PLP was observed to be completely or partially restored by various amino acids of histidine, phenylalanine, glycine, threonine, cysteine, serine and leucine, as well as valine and lysine[11]. It can be suggested, as indicated by Rippa et al.[11], that the bonding of PGA with the enzyme may be prevented by the formation of a Schiff base between the aldehydic group of PLP and $\varepsilon$-amino group of a lysine residue at the active center. In further studies on the restoration process, Km value of the enzyme restored partially by lysine or valine was found to be identical to that of the native enzyme in the presence of PLP at the same concentration as used for the inactivation. The
kinetic data appear to indicate that PLP may be released from the enzyme by such amino acids, and suggest that the release may be due to the bonding of PLP with such an amino acid at a different site of PLP from its bonding site with the enzyme.

References


