Some Properties of 2,3-Bisphosphoglycerate Phosphatase from Rabbit Masseters

by

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Summary

1. 2,3-Bisphosphoglycerate phosphatase was partly purified by CM-cellulose column chromatography.
2. The enzyme activity was stable in the 50°C case of preincubation up to 30 min. By the addition of 4 mM 2-phosphoglycolate to the preincubation mixture activity remained perfect up to 30 min in the 55°C case of preincubation.
3. This enzyme was inhibited by low concentrations of p-chloromercuribenzoate and heavy metal ions. These results suggest that this enzyme is an SH-enzyme.
4. The reaction mechanism was of a ping-pong type.
5. Inhibition types of dihydroxyacetone phosphate and glyceraldehyde phosphate were uncompetitive against 2,3-bisphosphoglycerate and competitive against 2-phosphoglycolate.

Keywords: 2,3-bisphosphoglycerate phosphatase, rabbit masseter, muscle, kinetics.

Introduction

The synthesis and breakdown of 2,3-bisphosphoglycerate (2,3-BPG) are controlled by bisphosphoglycerate mutase and 2,3-bisphosphoglycerate phosphatase (2,3-BPG Pase) respectively^[1-6].

It is important to control the concentration of 2,3-BPG in the tissues in order to regulate phosphoglyceromutase (PG mutase) activity in glycolysis. Furthermore, some kinetic functions of 2,3-BPG Pase, besides the primary function, are correlated to those of PG mutase.

Although MAENO et al.^[5,6] reported some properties of 2,3-BPG Pase in rabbit masseters, the kinetics and enzymatic characterization of 2,3-BPG Pase are not well-known. As a study to clarify 2,3-BPG metabolism, this paper describes the reaction mechanism, thermostability and kinetics against inhibitors of 2,3-BPG Pase in rabbit masseters.

Materials and Methods

Reagents

The following reagents were purchased from Boehringer Mannheim Co.: 2,3-

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BPG, adenosine 5'-triphosphate (ATP), phosphoglycerate kinase (PGK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), reduced nicotinamide adenine dinucleotide (NADH), dihydroxyacetone phosphate (DAP), glyceraldehyde phosphate (GAP) and glutathione. Other reagents were commercial products of the highest grade available.

Enzyme Extraction

Rabbit masseters were cut out from the mandible using a razor knife and minced on a glass plate set on an ice block. The minced masseters were homogenized with 1 mM HEPES buffer (pH 7.0) in the ratio of 100 mg to 1.0 ml and the homogenate was centrifuged at $10,000 \times g$ for 15 min to obtain the supernatant fraction of the tissues.

Partial Purification by CM-Cellulose Column Chromatography

The supernatant was applied to a CM-cellulose column (1×10 cm) equilibrated with the above buffer and eluted by adding 50 ml each of 1, 10, 50, 70 and 100 mM HEPES buffer (pH 7.0) in succession. The eluate was collected to 5 ml per fraction.

Determination of Enzyme Unit of PGK and GAPDH

Enzyme activity of these enzymes was determined by using the methods recommended by Boehringer Mannheim Co. One unit of these enzymes was represented by the international unit.

Assay for 2,3-BPG Pase Activity

Enzyme activity was determined by measuring the reaction product, inorganic phosphate (Pi) or 3-phosphoglycerate (3-PG). The enzyme assay mixture to measure Pi contained 0.25 mM 2,3-BPG, 2 mM 2-phosphoglycolate, 50 mM HEPES buffer (pH 7.4) and the enzyme preparation in a final volume of 0.4 ml. After the reaction mixture was incubated at 37°C for 30 min, 0.4 ml of 2.5 N H_2SO_4 was added to the mixture to terminate the enzyme reaction and to measure Pi derived from the substrate using Bartlett's method^[7]. The amount of phosphorus (μmol) formed by this enzyme reaction was considered to be the activity of 2,3-BPG Pase. This procedure was only used for examining the effect of metal ions.

To measure 3-PG, one of the reaction products, the following reagents were furthermore added to the above reaction mixture: 0.75 mM ATP, 1.25 mM MgSO_4 , 0.1 mM NADH, and 1.0 unit of PGK and GAPDH. The enzyme reaction was continued for 30 min at 37°C and terminated by adding 0.4 ml of 10 % perchloric acid. This acidic solution destroyed NADH which remained in the above reaction mixture. Subsequently, 2.0 ml of 10 N NaOH containing 0.01 % hydrogen peroxide was added to the above mixture. This strong alkaline solution (2.8 ml) was further incubated for 1 hr at 37°C to derive the fluorescent substance NAD, which was formed by coupling enzymes and was equivalent to 3-PG. The fluorescence originating from NAD was measured with a fluorescence spectrophotometer at an excitation wavelength of 365 nm and an emission wavelength of 470 nm. The amount of formed NAD (μmol) corresponding to 3-PG was considered to be the value of 2,3-BPG Pase activity.

Results

Partial Purification of 2,3-BPG Pase by CM-Cellulose Column Chromatography

The elution profile of 2,3-BPG Pase activity is illustrated in Fig. 1. The major 2,3-BPG Pase activity was eluted as a single peak with 50 mM HEPES buffer (pH 7.0) and the yield of this enzyme activity was approximately 90 % of the crude extracts (data not shown). The active fraction served as the enzyme preparation.

Thermostability of 2,3-BPG Pase

To examine the thermostability of this enzyme, the enzyme preparation was preincubated under the conditions described in the legend of Fig. 2. In the 50°C case of incubation, enzyme activity was stable after 30 min but at 55°C 30 % of the original activity of the enzyme preparation was lost after 30 min. At 60°C, 70 % of the original activity was lost after 5 min.

In the case of the addition of 4 mM 2-phosphoglycolate into the above preincubation mixture enzyme activity was not lost after 30 min at 55°C (Fig. 3).

Inhibition by p-Chloromercuribenzoate

The enzyme preparation was preincubated with or without p-chloromercuri-

Fig. 1 CM-cellulose column chromatogram of 2,3-BPG Pase activity. The enzyme preparation was eluted from a CM-cellulose column (void volume: 8 ml) by adding five different concentrations of HEPES buffer (pH 7.0) in succession at a flow rate of 50 ml/hr. Each fraction (5 ml) was assayed for 2,3-BPG Pase activity by the method described in "Materials and Methods."

Fig. 2 Thermostability of 2,3-BPG Pase. The preincubation mixture contained 100 mM HEPES buffer (pH 7.4), 0.0025% bovine serum albumin and the enzyme preparation in 0.2 ml. The mixture was incubated at 50, 55 or 60°C for every 5 min until 30 min respectively. After preincubation the mixture was immediately cooled down in an ice bucket, and the enzyme reaction was normally continued as described in "Materials and Methods." The data are represented as a percentage of the remaining enzyme activity against the original activity of the enzyme preparation; ○, 50°C; ●, 55°C; ▽, 60°C.

benzoate (pCMB) as described in the legend of Fig. 4, and then the enzyme reaction was normally continued for 30 min. Enzyme activity was gradually inhibited by increasing the concentrations of pCMB. In the case of 1.5×10^{-7} M pCMB, enzyme activity was inhibited by 45 % of the original activity.

Reactivation of Enzyme Activity by the Addition of SH-Reagents

After preincubation of the enzyme preparation with 1.5×10^{-7} M pCMB, the preincubation mixture was further incubated at 37°C for 5 min with some SH-reagents as described in the legend of Fig. 5. Enzyme activity was recovered up to 90–100 % of the original activity by adding 10^{-4} M of the above SH-reagents respectively.

Effect of Metal Ions

The enzyme reaction was regularly continued with or without metal ions under the conditions of the legend of Table 1. Enzyme activity was inhibited by the addi-

Fig. 3 Effect of 2-phosphoglycolate on the thermostability of 2,3-BPG Pase. The enzyme preparation was preincubated with or without 4 mM 2-phosphoglycolate under the same buffer solution as described in the legend of Fig. 2. After preincubation at 55°C at different intervals, 5 min up to 30 min, the enzyme reaction was normally continued. The data are represented in the same manner as in Fig. 2; □, with 4 mM 2-phosphoglycolate; ●, without 2-phosphoglycolate.

Fig. 4 Inhibition of p-chloromercuribenzoate. The preincubation mixtures including 100 mM HEPES buffer (pH 7.4), 0.0025% bovine serum albumin and the enzyme preparation were incubated with some different concentrations of p-chloromercuribenzoate (pCMB) at 37°C for 5 min. After preincubation, the enzyme reaction was normally continued. The data are represented as the relative activity (%) to the activity in the case of no inhibition.

Fig. 5 Reactivation from the pCMB inhibition by SH-reagent. After preincubation of the enzyme preparation with 1.5×10^{-7} M pCMB as described in the legend of Fig. 4, the mixture was further incubated at 37°C for 5 min with some different concentrations of L-cysteine (●), glutathione (○) or mercaptoethanol (▽), and then the enzyme reaction was normally continued. The data are represented as the relative activity (%) to the original activity.

Table 1. Effect of metal ions. Each metal ion was added to the enzyme reaction mixture at different concentrations as indicated in this table. The enzyme reaction continued normally as described in "Materials and Methods." The data are represented as the relative activity (%) to the original activity of the enzyme preparation.

Compounds (Mol)	10^{-6}	10^{-5}	10^{-4}	10^{-3}
CaCl ₂	100	100	100	—
BaCl ₂	100	100	100	—
MgCl ₂	100	100	100	100
ZnCl ₂	100	100	100	100
SrCl ₂	100	100	100	100
CdCl ₂	100	100	100	100
CoCl ₂	100	100	100	100
NiCl ₂	100	100	94	89
CuCl ₂	100	100	81	73
HgCl ₂	50	13	3	—
AgNO ₃	95	77	17	—

tion of NiCl₂, CuCl₂, HgCl₂ and AgNO₃, especially 10^{-6} M HgCl₂ which inhibited approximately 50 % of the original activity.

Kinetics of 2,3-BPG Pase Reaction

The kinetic profiles of the reaction mechanism were determined by depicting double reciprocal plots as described in the legend of Fig. 6. The parallel straight lines depicted in Fig. 6 show that the reaction mechanism is a ping-pong mechanism.

Further, high concentrations of 2,3-BPG (0.25 mM and above) and/or 2-phosphoglycolate (2.5 mM and above) inhibited enzyme activity (data not shown) and the straight lines depicted by the double reciprocal plots of the initial velocities of one substrate against another were focused at a spindle as shown in Fig. 7.

Inhibition Types of DAP and/or GAP against 2,3-BPG and/or 2-Phosphoglycolate

As DAP and GAP inhibited enzyme activity^[5], inhibition types of these reagents were examined by double reciprocal plots as shown in Figs. 8 and 9. The inhibition types of both chemicals were uncompetitive against 2,3-BPG (Fig. 8) and competitive against 2-phosphoglycolate (Fig. 9).

Discussion and Conclusions

2,3-BPG Pase from rabbit masseters was partly purified by CM-cellulose column chromatography as described by MAENO et al^[5,6].

Since this enzyme was eluted as a single peak from the CM-cellulose column with 50 mM HEPES buffer (pH 7.0), the fraction which contained the highest enzyme activity was used as the enzyme preparation.

To examine the thermostability of 2,3-BPG Pase, the enzyme preparation was preincubated with the assay buffer and bovine serum albumin. Thirty min after the preincubation at 55°C, enzyme activity of the enzyme preparation lost 30 % of the original activity, and 70 % of activity was lost after 5 min at 60°C. However, by the addition of 2-phosphoglycolate in the preincubation mixture, activity was stable up to 30 min in the case of the 55°C incubation. These results suggest that 2-phosphoglycolate is not only an activator of 2,3-BPG Pase as described by others^[1-5],

but also sustains enzyme structure.

Although this enzyme is generally an SH-enzyme in animals^[3,4], that of masseters is not known. Therefore, pCMB inhibition and the recovery from pCMB inhibition by the addition of SH-reagents were examined as shown in Figs. 4 and 5. As a result, this enzyme was inhibited by low concentrations of pCMB and recovered by adding SH-reagents; cysteine, glutathione and mercaptoethanol. Also, this enzyme was

Fig. 6 Dependence of the initial velocity of 2,3-BPG Pase on 2,3-BPG or 2-phosphoglycolate. (A) The reaction mixture contained the same reagents and enzyme preparation as indicated in "Materials and Methods," except that 2,3-BPG was used as indicated above, and 0.25 mM, 0.5 mM or 1.5 mM 2-phosphoglycolate was used respectively. (B) The reaction mixture contained the same as described in the legend of (A), except for 2-phosphoglycolate as indicated, and 12.5 μM , 25 μM or 50 μM 2,3-BPG. The data are represented by double reciprocal plots.

Fig. 7 The inhibition type of high concentrations of 2,3-BPG or 2-phosphoglycolate against each other. (A) The reaction mixture contained the same reagents as indicated in the legend of Fig. 6, except that higher concentrations of 2,3-BPG (0.25, 0.5, 0.75 or 1.0 mM) were used, and 2-phosphoglycolate was used as indicated in (A). (B) Higher concentrations of 2-phosphoglycolate (2.0, 3.75 or 5.0 mM) were used and 2,3-BPG was used as indicated in (B). The data are represented by double reciprocal plots.

inhibited by the heavy metal ions; NiCl_2 , CuCl_2 , HgCl_2 and AgNO_3 . These results show that 2,3-BPG Pase from rabbit masseters has an SH-group at the active center of 2,3-BPG Pase.

The relation between the concentrations of 2,3-BPG or 2-phosphoglycolate and the initial velocities was determined to elucidate the reaction mechanism of 2,3-BPG Pase. As shown in Fig. 6, the straight lines of the double reciprocal plots were parallel with the different concentrations of 2-phosphoglycolate and/or 2,3-BPG against each other chemical. This result suggests that this enzyme reaction pro-

Fig. 8 The inhibition type of DAP or GAP against 2,3-BPG. The enzyme reaction was continued as described in "Materials and Methods," except that 2,3-BPG concentrations were used as indicated in this figure, and DAP or GAP was added to the reaction mixture as indicated in (A) or (B) respectively. The data are represented by double reciprocal plots; the control was the values obtained by no addition of inhibitors.

Fig. 9 The inhibition type of DAP or GAP against 2-phosphoglycolate. An inhibitor and 2-phosphoglycolate were used as indicated in this figure, other reagents were added as described in "Materials and Methods." The data are represented by double reciprocal plots; the control was the same as in the legend of Fig. 8.

gressed as the ping-pong mechanism described by CLELAND^[8]. This is similar to the reaction mechanism of phosphoglyceromutase from rabbit dental pulp^[9], gingiva^[10,11] and masseters^[6].

As high concentrations of 2,3-BPG and 2-phosphoglycolate inhibited 2,3-BPG Pase^[3,4], inhibition types were determined by double reciprocal plots. Both substrate and activator, exceeding the optimal concentrations, showed mutually competitive types of inhibition as shown in Fig. 7. This result suggests that 2,3-BPG inhibition is caused by the binding of 2,3-BPG to the monophosphate binding site of a phospho-enzyme, and 2-phosphoglycolate inhibits enzyme activity by binding to the bisphosphate binding sites of a dephospho-enzyme. These results are similar to the substrate inhibition of phosphoglyceromutase^[3,4,6,9-11].

The inhibition types of DAP and GAP were examined by double reciprocal plots. The inhibition types of these reagents were uncompetitive with respect to 2,3-BPG and competitive with respect to 2-phosphoglycolate. These results suggest that after 2,3-BPG binds to its binding site and the enzyme becomes a phospho-enzyme, the inhibitors bind to the monophosphate binding sites where 2-phosphoglycolate should be to activate the enzyme. This is also similar to the inhibition types of these reagents to phosphoglyceromutase^[6].

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