BBA 66169

REQUIREMENT OF DIVALENT CATION FOR HUMAN PLATELET PHOSPHORYLASE ACTIVITY

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(Received May 14th, 1970)

SUMMARY

Human platelets contain a glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) which requires divalent cation (Mg^{2+}, Mn^{2+}, Sr^{2+}, Ca^{2+}) for activity. This phosphorylase can be obtained from both a soluble and particulate fraction following extraction and centrifugation at 105 000 $\times$ g. The particulate fraction is inactive in the absence of divalent cation. The K_m for Mg^{2+} and Ca^{2+} activation is 0.3 and 1.5 mM, respectively. Divalent cation activation is suboptimal and can be increased 2.5-fold following incubation with MgATP at 37° for 1 h. This enhanced activation is obtained with MgATP only. The particulate fraction, when fully activated, represents 60% of total platelet phosphorylase activity. It is associated with the platelet glycogen.

A 16-fold purified soluble phosphorylase can be inactivated in the presence of EDTA, and optimally reactivated following addition of Mg^{2+}. Sucrose gradient studies of the EDTA-inactivated enzyme reveal the presence of inactive monomer, dimer and tetramer species. Addition of Mg^{2+} alone, converts these inactive molecular species to both phosphorylase b (AMP dependent) and phosphorylase a.

INTRODUCTION

In previous communications[1,2], human platelet phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1) was characterized with respect to molecular weight, AMP dependence, and MgATP activation. A phosphorylase b and phosphorylase a dimer were found to be the predominant active molecular species, both having an apparent molecular weight of 177 000 and an $s_{20,w}$ of 8.9 S. The phosphorylase $b \rightleftharpoons$ phosphorylase a conversion was shown to take place in a 27 000 $\times$ g charcoal-treated extract following appropriate extraction and incubation in NaF and EDTA. Human platelets were shown to contain a phosphorylase which was "inactive" in the absence as well as presence of AMP. This inactive phosphorylase appeared to be present as monomer and dimer species which were activated with MgATP to both

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phosphorylase *b* (AMP dependent) as well as phosphorylase *a*. Phosphorylase activity was shown to be present in a 105 000 × *g* supernatant as well as particulate fraction. The particulate fraction was completely inactive in the absence of MgATP.

This particulate fraction has been further characterized. It has been shown to contain all of the platelet glycogen. It is activated by divalent cation alone (*i.e.* Mg^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}) as well as by MgATP. Divalent cation activation is suboptimal, and does not require prior incubation at 37° for 1 h. MgATP activation is optimal but requires prior incubation at 37° for 1 h.

A soluble phosphorylase extract has been purified 16-fold. The purified enzyme can be inactivated with EDTA to phosphorylase monomer, dimer and tetramer. These fractions can be activated with Mg^{2+} alone to phosphorylase *b* and phosphorylase *a*.

MATERIALS AND METHODS

Preparation of phosphorylase

27 000 × g crude extract. Freshly obtained human platelets were prepared as described previously[3,4]. The platelet pellet was rapidly frozen with liquid N_2, −196°, and ground to a fine powder with sand in a cold mortar and pestle. Then 3 vol. per g wet wt. of 0° buffer extractant containing 40 mM β-glycerolphosphate buffer (pH 6.8), 10 mM mercaptoethanol, 0.2 mM EDTA, were added. The mortar was thawed at room temperature. The thawed slurry was reground and transferred to a centrifuge tube which was sedimented at 4000 × *g* for 10 min. (All centrifugations were performed at 4°.) The sediment was discarded and the supernatant centrifuged at 27 000 × *g* for 10 min. The resultant supernatant was then treated with charcoal (neutralized Norit A), 40 mg per ml, to remove AMP, and recentrifuged at 27 000 × *g* for 10 min. This material provided the crude phosphorylase enzyme mixture which represented an average 3-fold purification over the 4000 × *g* extract (Table I). Repetition of the charcoal procedure did not result in any appreciable decrease of total phosphorylase activity or percentage of phosphorylase *a*.

*Partially purified phosphorylase**. This was prepared from the 27 000 × *g* charcoal extract. The extract was lyophilized and then dissolved in 1/10 its original volume of 10 mM mercaptoethanol. The insoluble material was discarded following centrifugation at 5000 × *g* for 5 min. The supernatant was diluted 1:2 in 10 mM mercaptoethanol and then adjusted to pH 7.5 by cautious addition of 5 μl aliquots of 1.0 M NaOH. The precipitate obtained was discarded following centrifugation as above. The resultant supernatant was 16-fold purified over the original 4000 × *g* extract (average of ten experiments) (Table I). As can be noted, charcoal extraction increased phosphorylase activity 1.5-fold. This is probably a result of removal of ATP and ADP. These adenine nucleotides have been shown to inhibit human platelet phosphorylase[2].

The residual supernatant was diluted 1:5 in a diluent which provided a final concentration of 40 mM β-glycerolphosphate (pH 6.8), 10 mM mercaptoethanol,

* The difficulty in obtaining large quantities of fresh human platelets (approx. 1 g wet wt. per 500 ml whole blood) precluded attempts at further purification of human platelet phosphorylase. Nevertheless, the prime role played by this tissue in hemostasis and its significant dependence on glycogenolysis[3,4], was considered significant justification for obtaining the data to be presented.

TABLE I

PARTIAL PURIFICATION OF HUMAN PLATELET PHOSPHORYLASE

Fraction	*Protein (mg/ml)*	*Enzyme activity (μmoles glucose 1-phosphate per ml per h)*	*Specific activity (μmoles/mg per h)*	*Purification*	*Yield (%)*
4000 × *g* homogenate	20.5	0.308	0.15	—	100
27 000 × *g* charcoal	9.6	0.432	0.45	3.0	150
Lyophilized–concentrated	39.1	47.6	1.22	8.1	200
pH-7.5 supernatant	18.8	45.8	2.44	16.2	180

20 mM NaF and 5–10 mM EDTA (this range of EDTA was required to give a negligible rate, in the absence of Mg^{2+}) prior to sucrose gradient analysis.

Particulate phosphorylase. The platelet pellet was frozen and extracted similar to that of above, except for a change in the composition of the extractant: 20 mM β-glycerolphosphate buffer (pH 6.8), 10 mM mercaptoethanol, 5 mM EDTA and 20 mM NaF. The resultant platelet extract was not treated with charcoal but was fractionated into two parts following centrifugation at 4000 × *g* for 10 min: a 105 000 × *g* supernatant and a 105 000 × *g* particulate fraction was obtained following centrifugation for 1 h. The particulate fraction was suspended with a Dounce homogenizer in its original volume of the same extractant (except for a change in the EDTA concentration from 5 to 0.2 mM).

Sucrose gradient analysis

Sucrose gradient analysis was performed on the partially purified enzyme by the method of MARTIN AND AMES[5]. Continuous gradients of 5–20% sucrose in buffer extractant containing 40 mM β-glycerolphosphate buffer (pH 6.8), 10 mM mercaptoethanol, 5 mM EDTA and 20 mM NaF were run in a Beckman Model L2 ultracentrifuge employing a 39 SW head with total volume of 275 μl carefully layered onto the gradient. Catalase, employed as marker protein, was assumed to have a molecular weight of 250 000, $s_{20,w} = 11.3$ S (ref. 6) and was assayed at 406 mμ. Gradients were centrifuged at 37 000 rev./min for 14 h at 4°. The gradient was tapped, and 3- or 4-drop aliquots were collected. This was diluted by addition of 300 μl of the above buffer extractant and allowed to sit at 30° for 30 min. Alternate tubes were then assayed for enzyme activity.

Assay of phosphorylase activity

Phosphorylase activity was measured in the direction of glycogen degradation at 30°. For sucrose gradient analysis, 300 μl was added to a cuvette made up to a final volume of 0.5 ml and containing 40 mM β-glycerolphosphate buffer, 20 mM NaF, 5 mM EDTA, 10 mM mercaptoethanol, 10 mM orthophosphate, 0.3 g glycogen per 100 ml, 0.46 mM $NADP^+$, 2 μg glucose-6-phosphate dehydrogenase, 10 μg phosphoglucomutase with or without 2 mM AMP and/or 20 mM MgCl. Phosphorylase activity of the particulate fraction was assayed with 20–50 μl of tissue extract with the same assay mixture as above, except for the addition of 0.2 mM EDTA rather than 5 mM EDTA. The particulate phosphorylase was assayed in the presence and absence of

5 mM AMP and/or 5 mM $MgCl_2$. Rates were zero in the absence of orthophosphate or glycogen. Rates were linear with time as well as with tissue extract.

Materials

Distilled deionized water was used at all times. All chemicals were reagent grade. Glucose-6-phosphate dehydrogenase, EDTA, $NADP^+$, ATP, AMP and cyclic AMP were obtained from Sigma Chemical Co. (St. Louis, Mo.). Phosphoglucomutase was obtained from Boehringer Mannheim Corp. (New York). Norite A was obtained from Fisher Scientific (New York), and shell fish glycogen was obtained from Mann Research Co. (New York). The glycogen was dissolved in distilled water and treated twice with charcoal, 60 mg/g glycogen. The glycogen in the adsorbed solution was then precipitated twice with absolute alcohol, dissolved in β-glycerol phosphate buffer, and assayed for glycogen content. Glycogen was assayed by the method of HASSID AND ABRAHAM[7]. Sephadex G-25 was obtained from Pharmacia (Piscataway, N.J.).

RESULTS

Particulate phosphorylase

With the addition of as little as 0.2 mM EDTA to the extractant solution, particulate phosphorylase activity was reduced to zero, in the absence as well as presence of 5 mM AMP. Addition of 5 mM divalent cation to the assay cuvette resulted in considerable phosphorylase activity. This was preceded by a lag period of 0–3 min. Mg^{2+}, Ca^{2+} and Sr^{2+} were similarly effective (Table II). Addition of 5 mM AMP to the cuvette increased the rate approx. 2-fold in the presence of Mn^{2+}, Ca^{2+} or Sr^{2+}, and 2.7-fold with Mg^{2+}.

When the particulate phosphorylase was incubated with 5 mM metal-ATP for 1 h at 37° and then assayed in the presence of 5 mM metal-ATP, no enhancement of rate was noted over Mn^{2+}, Ca^{2+} and Sr^{2+} alone. However, 5 mM MgATP increased the rate over Mg^{2+} alone, 2.5-fold. Addition of 5 mM AMP + 5 mM metal-ATP to the incubation mixture resulted in a small or negligible increase in activity over metal + AMP for Mn^{2+}, Ca^{2+} and Sr^{2+}. However, MgATP + AMP did result in a 1.6-fold

TABLE II

ACTIVATION OF INACTIVE PARTICULATE PHOSPHORYLASE WITH DIVALENT CATION AND MgATP

Additions to assay cuvette consisted of 5 mM AMP, 5 mM divalent cation or 5 mM MgATP. Values represent enzyme rate in μmoles glucose 1-phosphate per g wet wt./per h at 30° (average of four to five experiments).

Divalent cation	*Additions to assay cuvette:*					
	None	*AMP*	*Metal*	*Metal + AMP*	*Metal–ATP**	*Metal–ATP* + AMP*
Mg^{2+}	0	0	11.9	32.0	29.4	51.2
Mn^{2+}	0	0	12.1	22.6	11.8	28.1
Ca^{2+}	0	0	12.2	25.9	11.4	29.6
Sr^{2+}	0	0	12.9	26.3	11.9	28.4

* The enzyme was preincubated with metal–ATP for 1 h at 37° and then added to the assay cuvette containing metal–ATP or metal–ATP + AMP.

TABLE III

ASSOCIATION OF HUMAN PLATELET GLYCOGEN WITH PARTICULATE PHOSPHORYLASE

Fraction	*Protein (mg/g*)*	*Glycogen (μg/g)*	*Glycogen/ protein (μg/mg)*
Intact platelets	119	5598	47.0
4000 × *g* homogenate	58.3	2560	43.9
105 000 × *g* particulate	25.4	2656	104.3
105 000 × *g* supernatant	34.9	8	0.21

* g wet wt. of original intact platelets.

increase in phosphorylase activity over Mg^{2+} + AMP, and 4.3-fold increase in activity over Mg^{2+} alone*. When MgATP was added directly to the assay cuvette, in the absence of preincubation, the rate obtained was similar to that for Mg^{2+} alone (data not shown).

Association of glycogen with particulate phosphorylase

The 4000 × *g* platelet homogenate glycogen was fractionated into a soluble and particulate 105 000 × *g* fraction and analyzed for glycogen. All of the glycogen was found to be present in the particulate fraction obtained from this homogenate (Table III).

Kinetics of divalent cation activation

The kinetics of Mg^{2+} and Ca^{2+} activation of particulate phosphorylase, in the presence and absence of 5 mM AMP is depicted in Fig. 1. The K_m for Mg^{2+} was 0.3 mM or 1/5 the K_m for Ca^{2+}, which was 1.5 mM. Mg^{2+} + AMP increased v_{max} 2.7-fold. Ca^{2+} + AMP lowered the K_m for Ca^{2+} alone 3.6-fold and raised the v_{max} 1.6-fold.

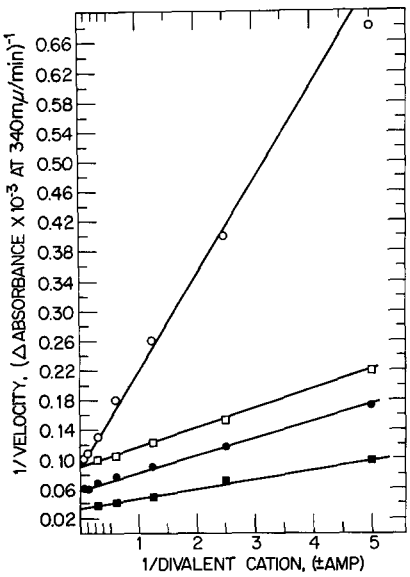
Sucrose gradient analysis of inactive partially purified phosphorylase

The soluble phosphorylase could be inactivated in the presence of sufficient EDTA and optimally reactivated by the addition of Mg^{2+}. This permitted further characterization of the divalent cation requirement, with respect to the possible activation of inactive molecular species. The soluble enzyme was purified 16-fold, inactivated with 5–10 mM EDTA and placed on two sucrose gradients run simultaneously[2]. Following tapping of the gradients, alternate tubes were assayed for phosphorylase activity in the presence and absence of 10–20 mM Mg^{2+} for both gradients. One gradient was run in the presence of 2 mM AMP, the other in its absence.

40 mM β-glycerolphosphate buffer

Fig. 2 is representative of three different experiments. The apparent average molecular weight and $s_{20,w}$ value for the monomer, dimer and tetramer species were respectively: 81 750 (5.4 S), 186 500 (9.3 S) and 378 000 (14.8 S). This figure presents

* Addition of 0.4 mM adenosine 3′,5′-cyclic monophosphate had no effect on the rate of the untreated enzyme or the enzyme treated with Mg^{2+} or MgATP (in the presence or absence of 37° incubation) at pH 6.8 or 8.2. Similar negative results were obtained for the partially purified soluble enzyme, before or after passage through a Sephadex G-25 column.



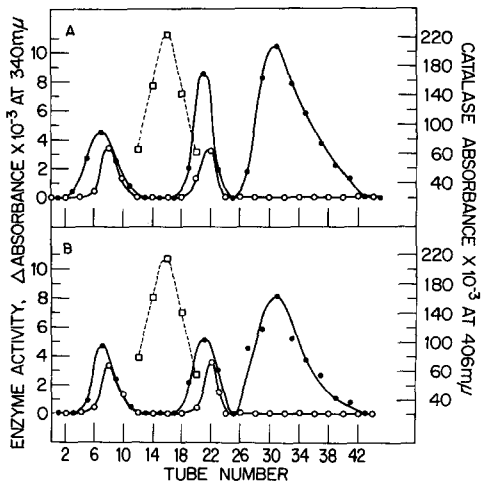


Fig. 1. Lineweaver–Burke plot for interaction of divalent cation ± 5 mM AMP with particulate phosphorylase. The four lines represent, ○, $CaCl_2$; ●, $CaCl_2$ + AMP; □, $MgCl_2$; ■, $MgCl_2$ + AMP.

Fig. 2. Sucrose gradient analysis of 16-fold purified phosphorylase inactivated with 5 mM EDTA. The gradient was tapped and 3- or 4-drop aliquots were collected. This was diluted by addition of 300 μl of buffer extractant containing 5 mM EDTA and allowed to sit at 30° for 30 min. A and B refer to enzymatic measurements made in the presence and absence of 2 mM AMP, respectively. Alternate tubes were assayed for phosphorylase activity in the absence (○—○) and presence (●—●) of 10 mM $MgCl_2$. Catalase protein was determined from the absorbance of 406 mμ (□--□).

evidence for the presence of inactive phosphorylase monomer, dimer and tetramer in the absence of Mg^{2+}. The addition of Mg^{2+} (divalent cation alone) gave results similar to that previously reported for MgATP activation[1,*]. Thus from Fig. 2B, wherein activity was measured in the absence of AMP, inactive phosphorylase monomer must have been converted to AMP-independent phosphorylase *a*. Similarly, phosphorylase *a* dimer and tetramer activity increased in the presence of Mg^{2+}, indicating activation of phosphorylase *a* dimer and tetramer.

From Fig. 2A, wherein activity was measured in the presence of AMP, inactive monomer must have been converted to phosphorylase *b* (as well as phosphorylase *a*) since the integrated area of the monomer peak of Fig. 2A is 1.3-fold greater than the integrated area of the monomer peak of Fig. 2B. Similarly, inactive dimer and tetramer must have been converted to active phosphorylase *b* (as well as phosphorylase *a*) since the net integrated areas of the dimer and tetramer peaks obtained in the presence of Mg^{2+} (Fig. 2A) were 1.6- and 1.9-fold greater, respectively, than the integrated areas of the respective peaks in Fig. 2B. In the absence of Mg^{2+} the integrated areas of the dimer and tetramer peaks were approximately the same for Figs. 2A and 2B.

* It is very likely that the MgATP activation reported previously with sucrose gradient studies was predominantly Mg^{2+} activation, since the Mg^{2+} and ATP concentrations were 10 and 1 mM, respectively[1].

DISCUSSION

These data reveal the presence of soluble as well as particulate glycogen phosphorylase in human platelets. The particulate phosphorylase is evidently associated with all of the platelet glycogen. It is completely inactive in the absence of divalent cation. Activation by divalent cation is suboptimal and fairly rapid whereas activation by MgATP is optimal and requires prior incubation at 37° for 1 h (ref. 1). Following maximal activation, the particulate phosphorylase represents 60% of total phosphorylase activity. These data suggest that human platelet phosphorylase is structurally organized and closely associated with platelet glycogen. Similar structural organization has been reported for liver phosphorylase[8].

The mechanism of activation of inactive phosphorylase with divalent cation alone is of interest, since it has not been reported for other glycogen phosphorylases. It is clearly different from the mechanism of MgATP activation. The divalent cation activation is rapid and independent of ATP concentration. Inactive monomer, dimer and tetramer species become activated to phosphorylase *b* as well as phosphorylase *a* following divalent cation activation. These inactive species appear to require divalent cation for functional conformation and/or subunit association.

The slower MgATP activation of the particulate enzyme is operational for Mg^{2+} cation alone, not for the other cations tested. The MgATP activation is similar in some respects to that reported for the liver enzyme[9–11], but bifferent in other respects. It is attractive to consider the possibility that MgATP activation might be mediated by the activation of phosphorylase kinase[12–15]. However, the experimental data do not support this explanation: (1) The particulate enzyme was inactive in the presence or absence of AMP (Table II), whereas the inactive liver enzyme has 15–20% of the activity of active liver phosphorylase[10]. Furthermore, phosphorylase *b* could not have been present. (2) Addition of adenosine 3′,5′-monophosphate did not activate the enzyme, nor enhance activity in the presence of Mg^{2+} or MgATP at pH 6.8 or 8.2.

In regard to divalent cation requirement, the enzyme is different from other glycogen phosphorylases. However, the enzyme does resemble other glycogen phosphorylases[16–20] with respect to molecular weight[2], subunit structure[1,2], AMP dependence[1,2], substrate requirement[2] and ATP inhibition[2].

ACKNOWLEDGMENT

The authors are indebted to The New York Blood Center for their excellent cooperation in the supply of fresh human platelet-rich plasma. This investigation was supported by a grant-in-aid from the New York Heart Association.

REFERENCES

1 S. KARPATKIN AND R. M. LANGER, *J. Biol. Chem.*, 244 (1969) 1953.
2 S. KARPATKIN AND R. M. LANGER, *Biochim. Biophys. Acta*, 185 (1969) 350.
3 S. KARPATKIN, *J. Clin. Invest.*, 46 (1967) 409.
4 S. KARPATKIN AND R. M. LANGER, *J. Clin. Invest.*, 47 (1967) 2158.
5 R. G. MARTIN AND B. N. AMES, *J. Biol. Chem.*, 236 (1961) 1372.
6 J. B. SUMNER AND N. GRALEN, *Science*, 87 (1938) 284.
7 W. Z. HASSID AND S. ABRAHAM, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 34.

8 A. A. BARBER, S. A. ORRELL JR. AND E. BUEDING, *J. Biol. Chem.*, 242 (1967) 4040.
9 E. W. SUTHERLAND AND W. D. WOSILAIT, *J. Biol. Chem.*, 218 (1956) 459.
10 W. D. WOSILAIT AND E. W. SUTHERLAND, *J. Biol. Chem.*, 218 (1956) 469.
11 T. W. RALL, E. W. SUTHERLAND AND W. D. WOSILAIT, *J. Biol. Chem.*, 218 (1956) 483.
12 E. G. KREBS, D. J. GRAVES AND E. H. FISCHER, *J. Biol. Chem.*, 234 (1959) 2867.
13 E. G. KREBS, D. S. LOVE, G. E. BRATVOLD, K. A. TRAYSER, W. L. MEYER AND E. H. FISCHER, *Biochemistry*, 3 (1964) 1022.
14 W. L. MEYER, E. H. FISCHER AND E. G. KREBS, *Biochemistry*, 3 (1964) 1033.
15 C. A. CHELALA AND H. N. TORRES, *Biochem. Biophys. Res. Commun.*, 32 (1968) 70.
16 D. L. DEVINCENZI AND J. L. HEDRICK, *Biochemistry*, 6 (1967) 3489.
17 A. A. GREEN, G. T. CORI AND C. F. CORI, *J. Biol. Chem.*, 142 (1942) 447.
18 P. J. KELLER AND C. F. CORI, *Biochim. Biophys. Acta*, 12 (1953) 235.
19 N. B. MADSEN AND C. F. CORI, *J. Biol. Chem.*, 223 (1956) 1055.
20 H. E. MORGAN AND A. PARMEGGIANI, *J. Biol. Chem.*, 239 (1964) 2440.