BBA 66357

PURIFICATION AND PROPERTIES OF GLYCOGEN PHOSPHORYLASE FROM BOVINE SPLEEN

ATSUMI KAMOGAWA AND TOSHIO FUKUI
The Institute of Scientific and Industrial Research, Osaka University, Suita, Osaka (Japan)

(Received February 23rd, 1971)

SUMMARY

1. The inactive form of glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) was purified about 180-fold in 11% yield from bovine spleen. The procedure included adsorption on starch, treatment with α-amylase and Sephadex G-200 chromatography.

2. The purified enzyme revealed apparent homogeneity upon ultracentrifugation ($s_{20,w}$ = 8.5 S) and polyacrylamide gel electrophoresis with a tendency to form active aggregates. The molecular weight determined using Sephadex G-200 was approx. 190 000 and did not change upon enzymic conversion to the active form. The spleen enzyme contained 1.03 moles of pyridoxal-5'-*P*/100 000 g of protein. Its amino acid composition was similar to that of rabbit muscle and pig liver phosphorylase.

3. The inactive enzyme displayed activity only in the presence of AMP, and its activity increased about 4-fold upon conversion to the active form. Glucose-6-*P* acted as a competitive suppressor against AMP activation. Activation was also attained with cysteine, Na_2SO_4 and NaF.

4. From these results, it was assumed that spleen phosphorylase, as well as the kidney enzyme, is a type intermediate between muscle and liver phosphorylase.

INTRODUCTION

In mammalian tissues, glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) is distributed widely and abundantly, playing important roles in glycogen metabolism. Phosphorylase isolated from skeletal muscle and from liver exists in two interconvertible forms: phosphorylated (*a* or active) and dephosphorylated (*b* or inactive). There are distinct differences in the properties of the two forms. Muscle phosphorylase *b* displays nearly full activity in the presence of AMP (ref. 1) whereas inactive liver phosphorylase shows little or no activity under similar conditions[2]. Conversion of muscle phosphorylase *b* to *a* is accompanied by doubling of the molecular weight[3], in contrast with liver phosphorylase whose sedimentation constant does not change upon interconversion[4].

Abbreviation: DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid).

Phosphorylase from adrenal cortex[5], corpus luteum[6], leukocyte[7–9] and chloroma[10,11] appears to resemble liver phosphorylase, whereas the enzyme from heart[12,13], tracheal muscle[14] and brain[15–18] has properties similar to those of the enzyme from skeletal muscle. Phosphorylase from uterine smooth muscle[19] resembles the skeletal muscle enzyme in its activation by AMP, but a dimerization of the *b* form is not involved in the formation of the *a* form. Kidney phosphorylase[20] was reported to be a form of the enzyme different from both liver and muscle phosphorylase. Although spleen contains glycogen and its metabolizing system, no study on phosphorylase from this tissue has been reported.

This paper deals with the isolation of inactive spleen phosphorylase, its molecular and catalytic properties as well as conversion to the active form. The results show that spleen phosphorylase has properties of both the muscle and liver enzymes.

MATERIALS AND METHODS

Glucose-1-*P*, glucose-6-*P*, AMP, ATP, yeast alcohol dehydrogenase and bovine liver catalase were purchased from Sigma. L-Cysteine, soluble starch, sodium dodecyl sulfate and pyridoxal-5′-*P* were purchased from Wako. Shellfish glycogen and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Nakarai. Waxy rice starch was prepared by steeping waxy rice in dilute alkali followed by washing with water. Human γ-globulin, bovine serum albumin and ovalbumin were products of Mann. Crystalline α-amylase (EC 3.2.1.1) from *Bacillus subtilis* was a gift from Nagase. Sephadex G-200 and blue dextran 2000 were purchased from Pharmacia. All other chemicals were commercial products of reagent grade. The solutions of glucose-1-*P*, glucose-6-*P*, AMP, ATP and cysteine were adjusted to pH 6.7 before use.

Enzyme assay

Phosphorylase activity was measured in the direction of glycogen synthesis. The standard reaction mixture for inactive phosphorylase assay contained an appropriate amount of enzyme, 0.1 ml of glycerophosphate buffer (0.2 M, pH 6.7), 0.1 ml of glycogen (5%), 0.16 ml of glucose-1-*P* (0.1 M, pH 6.7), 0.05 ml of cysteine·HCl (0.3 M, pH 6.7), 0.02 ml of AMP (0.1 M, pH 6.7) and water in a total volume of 1 ml. Reaction was started by the addition of glucose-1-*P*. After incubation for 10 min at 30°, the reaction was stopped by the addition of 0.5 ml of trichloroacetic acid (5%), and the precipitate was removed by centrifugation. A 0.5-ml aliquot of the supernatant solution was removed for analysis of P_i by the method of FISKE AND SUBBAROW[21]. Protein concentration was determined by the method of LOWRY *et al.*[22] using bovine serum albumin as a standard, except in the determination of pyridoxal-5′-*P* and reactive sulfhydryl groups, in which it was measured by spectrophotometry using an absorbance index $A^{1\%}_{278\ nm} = 11.5$. The unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of P_i per min at 30° under the assay conditions. Specific activity is expressed as units/mg of protein.

Disk electrophoresis

Polyacrylamide disk electrophoresis was carried out according to the procedures of ORNSTEIN AND DAVIS[23] and of DAVIS *et al.*[12]. Phosphorylase activity in the gel after running at 4° and 3 mA/tube was detected as follows. The gels were in-

cubated for 30 min at 30° in a solution similar to that in the assay system. After incubation, the gels were washed with water and soaked in 0.01 M I_2–KI. Blue bands appeared at the site of phosphorylase activity. Protein was stained with Amido black.

Molecular weight estimation

The molecular weight of spleen inactive phosphorylase was estimated by the method of WHITAKER[24] using Sephadex G-200. Sephadex G-200 was allowed to swell for a week in 5 mM Tris–HCl buffer (pH 7.4) at room temperature, and then poured into a column (1.5 cm × 90 cm, Pharmacia) at 2°. Samples (5 mg protein in 1 ml) were carefully layered above the Sephadex. Elution was carried out at 2° using the same buffer, and 1.56-ml fractions were collected. Elution volumes were measured to the peak of absorption at 280 nm. Blue dextran 2000 was used to determine the void volume. The final values were calculated from several determinations.

Determination of absorbance index

The enzyme from Sephadex G-200 chromatography was dialyzed at 4° for 43 h against 2 l of 0.01 M Tris–HCl–2 mM mercaptoethanol buffer (pH 7.4) and used for measurements of absorbance with a Hitachi-Perkin-Elmer 139 spectrophotometer. For the determination of the absolute amount of protein, aliquots of the dialyzed solution were pipetted into weighed bottles and dried for 5 h at 70° in air and subsequently for 3 days at 70–85° under vacuum. Aliquots of the buffer were treated in the same manner as the enzyme sample to correct for the weight due to the buffer.

Determination of pyridoxal-5'-P

Enzyme solutions, each containing 3 and 5 mg protein, were mixed with performic acid to make a final concentration of 0.3 M, and the mixtures were kept for 30 min at room temperature to release the cofactor from the protein. After centrifugation for 15 min at 3000 × *g*, the supernatant solutions were analyzed for pyridoxal-5'-*P* by the phenylhydrazine method of WADA AND SNELL[25], by using an absorbance index of 27 200 $M^{-1} \cdot cm^{-1}$ for pyridoxal-5'-*P* phenylhydrazone.

Amino acid composition

Spleen phosphorylase was extensively dialyzed for 72 h against three changes of 3 l of distilled water, lyophilized and dried under vacuum at room temperature over H_2SO_4. Weighed amounts of the sample were hydrolyzed in 5.6 M HCl under vacuum at 110° for 24 or 48 h. After removal of the HCl, aliquots were analyzed by the procedure of MOORE AND STEIN[26] on a Hitachi 034 liquid chromatograph. The final values for each amino acid were calculated from averages or extrapolated values (serine and threonine).

Reactivity of sulfhydryl groups

The enzyme from Sephadex G-200 chromatography was extensively dialyzed at 4° for 5 days against four changes of 2 l of 0.01 M Tris–HCl buffer (pH 7.4) to remove mercaptoethanol. The reaction mixture contained 0.88 mg phosphorylase, 0.02 M glycerophosphate and 0.6 or 2.5 mM DTNB in a total volume of 1 ml. Incubation was carried out at pH 6.8 and 20°. The reaction was followed by measuring the absorbance at 412 nm, and 20-μl aliquots were removed at various time intervals

for the assay of enzymic activity without cysteine. The number of sulfhydryl groups reacted was calculated by using an absorbance index of 13 600 $M^{-1} \cdot cm^{-1}$.

Conversion to the active form

Purified inactive spleen phosphorylase (9 mg) was incubated with 10 mM ATP, 20 mM $MgCl_2$, 50 mM NaF and the 20–40% saturated $(NH_4)_2SO_4$ fraction (34 mg protein) of the crude extract from bovine spleen, in 2 ml of 25 mM Tris–HCl buffer (pH 7.8) at 30° for 60 min. The reaction mixture was subsequently chromatographed on a Sephadex G-200 column (2.5 cm × 100 cm) which had been equilibrated with 0.01 M Tris–HCl buffer (pH 7.4) containing 2 mM mercaptoethanol and 0.1 M NaF. Protein was eluted with the same buffer. Most of the protein appeared near the void volume, and the activities without AMP in following fractions. The active fractions were pooled and concentrated with a collodion bag at 4°, and used as the active form of spleen phosphorylase.

RESULTS

Purification procedure

Preparation of crude extract. Fresh bovine spleens obtained at a slaughterhouse were immediately chilled in ice and dissected free of fats and medullary tissues. The materials can be stored frozen at −20° for months without appreciable loss of enzymic activity. All subsequent steps were carried out at 2–6°, and centrifugations at 9000 × *g* for 10–15 min. The fresh or frozen tissues (1.3 kg) were homogenized for 1 min in a Waring blendor with 2 vol. of cold 0.01 M Tris–HCl buffer (pH 7.4) containing 2 mM mercaptoethanol (Buffer A). The homogenate was kept for 30 min at 2° to allow conversion of the active enzyme to the inactive form, and then centrifuged to remove the insoluble materials.

Adsorption on starch. The crude extract (2.5 l) was poured at 0° into 5 l of 2% waxy rice starch, which had been autoclaved for 60 min at 120° in Buffer A, as applied for the purification of potato phosphorylase[27]. While stirring was continued for a further 10 min at 1°, 960 ml of ethanol (−20°) were gradually added to the solution. The precipitate formed was collected by decantation and dissolved in 1250 ml of cold Buffer A by stirring overnight at 2°. The starch–enzyme complex was reprecipitated from this solution by adding 160 ml of ethanol (−20°) gradually at 0°. The precipitate formed was collected by decantation and dissolved again in 1250 ml of Buffer A by stirring for 4 h at 2°. This step was repeated once more in the same way.

Amylase treatment. To the starch–enzyme solution from the above step was added 1.25 mg of crystalline α-amylase at 2°. After incubation for 90 min at 2° under stirring, 302 g of solid $(NH_4)_2SO_4$ were added, and stirring was continued for a further 20 min. The precipitate formed after standing overnight at 2° was removed by centrifugation. To the clear supernatant solution were gradually added 452 g of solid $(NH_4)_2SO_4$. The solution was kept stirred for a further 40 min at 2°, and the precipitate formed was collected by centrifugation and dissolved in 10 ml of cold Buffer A.

Sephadex G-200 chromatography. The solution from the above step was centrifuged to remove insoluble material, if any, and applied onto a column of Sephadex

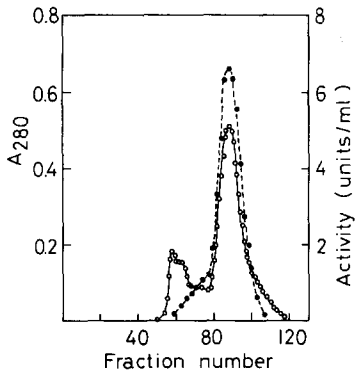


Fig. 1. Elution pattern of inactive bovine spleen phosphorylase from Sephadex G-200 column chromatography. The $(NH_4)_2SO_4$ fraction, containing 92 mg protein, was applied to a 5 cm × 106 cm column and eluted as described in the text; 10-ml fractions were collected. ○—○, absorbance at 280 nm; ● - - - ●, enzymic activity.

G-200 (5 cm × 106 cm) which had been washed with Buffer A. The column was eluted with the same buffer at 4°, and 10-ml fractions were collected. Inactive spleen phosphorylase appeared as a main peak in fractions No. 80–95 (Fig. 1). A small peak which appeared before the main one displayed weak enzymic activity, and perhaps contained aggregates of the enzyme. Fractions No. 81–98 were pooled and concentrated to 2.1 ml with a collodion bag at 4°. This was used as the enzyme sample in the following experiments. Table I gives a summary of the purification steps. A rather high activity without AMP in the crude extract might be due to the presence of the active form of phosphorylase. The ratio of the activity without AMP to that with AMP decreased during purification, and the final preparation gave a value of 0.03. It is not clear at present whether this value came from the contamination of the active enzyme or AMP or from the intrinsic property of the inactive enzyme. Specific activity calculated from the activity with AMP increased 180-fold during purification, and the yield was 11%. Although the last step (Table I) yielded no increase in specific activity, it was still effective in removing some impurities that occasionally appeared in the $(NH_4)_2SO_4$ precipitate.

TABLE I

PURIFICATION OF INACTIVE BOVINE SPLEEN PHOSPHORYLASE*

Fraction	*Total activity (units)*		*Total protein*	*Specific activity***	*Yield***
	−AMP	*+AMP*	*(mg)*	*(units/mg)*	*(%)*
Crude extract	2250	5550	65 000	0.085	100
1st starch precipitation	500	3430	2 350	1.46	62
2nd starch precipitation	150	2430	555	4.38	44
$(NH_4)_2SO_4$ precipitation	74	1420	92	15.5	26
Sephadex G-200 eluate	19	595	39	15.2	11

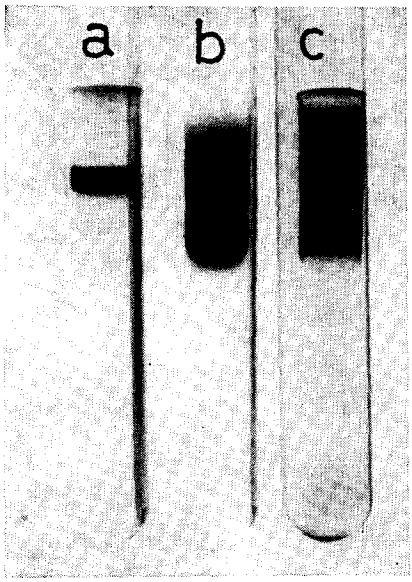
* Starting from 1.3 kg of fresh bovine spleens.
** Calculated from the total activity (+ AMP).

When the purified enzyme was subjected to rechromatography on a column of Sephadex G-200, a minor peak with enzymic activity again appeared before the main peak. This result supports a previous observation that the enzyme contained aggregated forms. Rechromatography of the purified enzyme on Sephadex led to considerable inactivation. The use of DEAE- or phospho-cellulose resulted in total loss of enzymic activity.

Stability. Purified spleen phosphorylase was rather unstable, especially in dilute solution. The enzyme lost 40% of its original activity during storage for a month at 2°. A freezing–thawing caused inactivation of the enzyme. No protective effect was observed with AMP, Mg^{2+}, cysteine or glycerol. The activity of inactivated enzymes was not restored by incubating with pyridoxal-5′-*P*.

Molecular properties

Homogeneity. The electrophoretic patterns of the purified enzyme are shown in Fig. 2. In addition to a large sharp band, about three faint bands with slower movements were observed in the gel (pH 8.9, 7.5%). Under these conditions, the gels displayed no enzymic activity. When electrophoresis was carried out in the gel (pH 7.9, 5%), the slowly moving bands became more noticeable, and all the bands exhibited phosphorylase activity. The slowly migrating components appeared to be aggregates of phosphorylase, as observed in the Sephadex G-200 chromatography. Similar aggregation has been observed in pig muscle phosphorylase *a* (ref. 28).



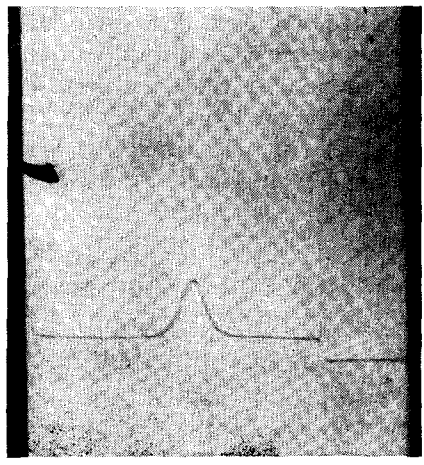


Fig. 2. Disk-electrophoresis of purified inactive bovine spleen phosphorylase. (a) Electrophoresis in 7.5% gel (pH 8.9)[10] followed by staining with Amido black; (b) electrophoresis in 5% gel (pH 7.9)[5] followed by staining with Amido black; (c) electrophoresis as in b and stained for enzymic activity. About 95 μg of enzyme protein was applied to each gel.

Fig. 3. Sedimentation pattern of purified inactive spleen phosphorylase at 10 mg/ml in 0.01 M Tris–HCl–2 mM mercaptoethanol buffer (pH 7.4). The picture shown was taken 44 min after a speed of 54 114 rev./min was reached at 2°.

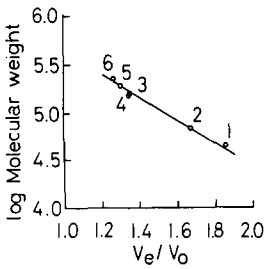


Fig. 4. Estimation of molecular weight of inactive spleen phosphorylase by Sephadex G-200 column (1.5 cm × 90 cm). 1, ovalbumin; 2, bovine serum albumin; 3, human γ-globulin; 4, yeast alcohol dehydrogenase; 5, inactive bovine spleen phosphoryalse; 6, bovine liver catalase. V_e and V_0 represent elution and void volumes, respectively.

Fig. 3 illustrates the sedimentation pattern of purified spleen phosphorylase. A single peak with $s_{20,w} = 8.5$ S was observed. This value is close to those for muscle phosphorylase *b* and liver inactive phosphorylase.

Molecular weight. The molecular weight of inactive spleen phosphorylase was determined by using Sephadex G-200. Fig. 4 shows a relationship between elution volumes and logarithms of the molecular weights for the enzyme and some reference proteins. An apparent molecular weight for inactive spleen phosphorylase was calculated to be 190 000, which is close to those for muscle phosphorylase *b* (ref. 29) and liver inactive phosphorylase[30].

Absorption spectrum and pyridoxal-5'-P. In 0.01 M Tris–HCl–2 mM mercaptoethanol (pH 7.4) buffer the enzyme showed a typical absorption spectrum of protein

TABLE II

AMINO ACID COMPOSITION OF INACTIVE BOVINE SPLEEN PHOSPHORYLASE

Amino acid	*g amino acid per 100 g protein*		
	*Spleen**	*Liver***	*Muscle****
Lysine	7.13	8.60	6.86
Histidine	2.69	4.62	3.23
Arginine	7.89	8.25	11.1
Aspartic acid	11.3	13.2	13.1
Threonine	3.99	4.31	4.07
Serine	3.42	4.42	3.10
Glutamic acid	12.8	13.4	15.0
Proline	3.42	4.54	4.89
Glycine	3.40	3.99	3.72
Alanine	4.98	5.98	5.81
Half cystine	—	1.57	0.99
Valine	5.73	7.40	7.18
Methionine	2.75	3.55	3.26
Isoleucine	5.13	6.85	6.36
Leucine	9.59	11.3	10.7
Tyrosine	5.37	4.90	6.63
Phenylalanine	5.29	6.97	6.35
Tryptophan	—	2.77	2.64

* Inactive bovine spleen phosphorylase (the present study).
** Inactive rabbit liver phosphorylase[30].
*** Rabbit muscle phosphorylase *b* (ref. 34).

with a maximum at 278 nm. The absorbance index at 278 nm, $E^{1\%}_{1\,cm}$, was calculated to be 11.5. At a higher concentration of the enzyme, a secondary maximum at 333 nm was observed. This might be due to the presence of bound pyridoxal-5'-*P* in the enzyme protein, as in other phosphorylases[31–33]. The absorbance index at 333 nm, $E^{1\%}_{1\,cm}$, was 0.565. Quantitative analyses by the phenylhydrazine method indicated the presence of 1.03 moles of pyridoxal-5'-*P* per 100 000 g of protein.

Amino acid composition. Table II presents the amino acid composition of inactive spleen phosphorylase. In general, its composition was similar to those of phosphorylases from rabbit muscle[34] and rabbit liver[30], which are included in the same table for comparison.

Reactivity of sulfhydryl groups. The number of reactive sulfhydryl groups of spleen phosphorylase was determined by the procedure of ELLMAN[35]. Fig. 5 gives the time courses of typical titrations of the enzyme with DTNB in the presence or the absence of sodium dodecyl sulfate, and of the enzymic activity remaining during the reaction. In its native state, 2.0 and 2.6 sulfhydryl groups per 100 000 g of protein were titrated within 60 min at the concentrations of the reagent, 0.6 and 2.5 mM, respectively. In the presence of sodium dodecyl sulfate, 6.8 sulfhydryl groups per 100 000 g of protein reacted with the reagent. The enzymic activity was fully retained after the reaction of approximately 3 sulfhydryl groups, and only partial inactivation

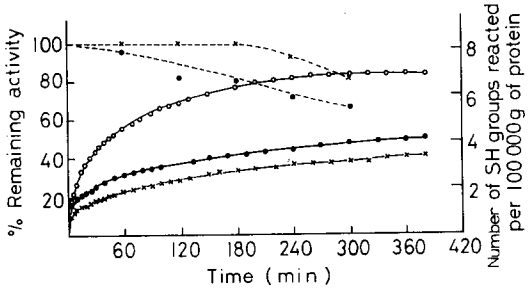


Fig. 5. Reactivity of the sulfhydryl groups of inactive spleen phosphorylase with DTNB. Enzyme (0.88 mg) was incubated with 0.6 or 2.5 mM DTNB in 0.02 M glycerophosphate buffer (pH 6.8) at 20°. For the denaturation of the protein, sodium dodecyl sulfate was added at a concentration of 1%, 1 min before th eaddition of DTNB. ●—● and ●- - -●, 2.5 mM DTNB; ×—× and × - - - ×, 0.6 mM DTNB; ○—○, 0.6 mM DTNB and 1% sodium dodecyl sulfate. The solid lines indicate the number of sulfhydryl groups reacted per 100 000 g of enzyme protein and the broken lines the remaining enzymic activities without cysteine.

was observed after 4 groups were reacted. These results differ from those for muscle phosphorylase *b* (ref. 36), which lost its activity completely with the reaction of 3 sulfhydryl groups per 92 500 g of protein with the reagent.

Catalytic properties. Optimal pH. Fig. 6 shows the pH–activity curve of inactive spleen phosphorylase. Maximal activity was observed at pH 6.7 when assayed in the presence of 2 mM AMP. Higher activities were obtained in glycerophosphate buffer than in Tris–maleate buffer throughout the pH range tested.

K_m for substrates. Lineweaver–Burk plots of the synthetic reaction of inactive

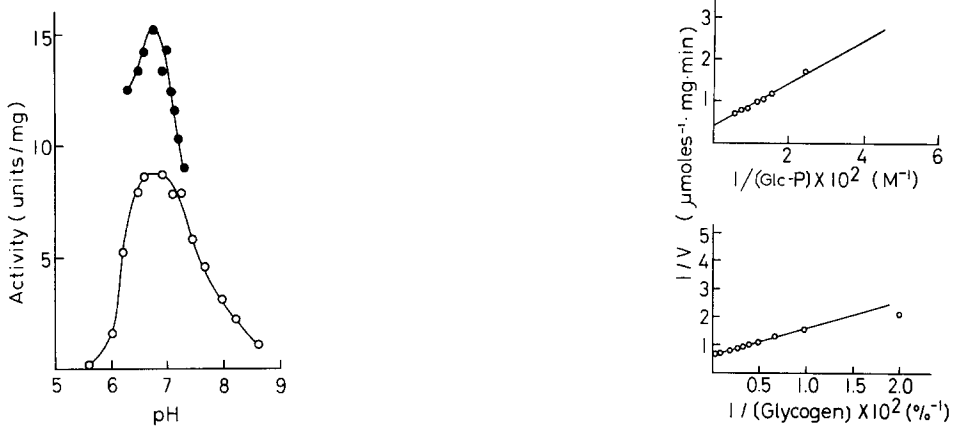


Fig. 6. Effect of pH on the reaction of inactive spleen phosphorylase. Enzyme (17 μg) was incubated for 10 min at 30° in a standard assay system using 0.01 M Tris–maleate or 0.02 M glycerophosphate buffers of various pH values as indicated. The final pH values of the reaction mixtures are shown. ○—○, Tris–maleate; ●—●, glycerophosphate.

Fig. 7. Effect of substrate concentration on the reaction of inactive spleen phosphorylase. Incubation was carried out in a standard assay system as described in MATERIALS AND METHODS, at various concentrations of one substrate, the concentration of the other substrate being maintained constant (16 mM and 0.5% for glucose-1-*P* and glycogen, respectively). The enzyme concentration was 17 μg/ml.

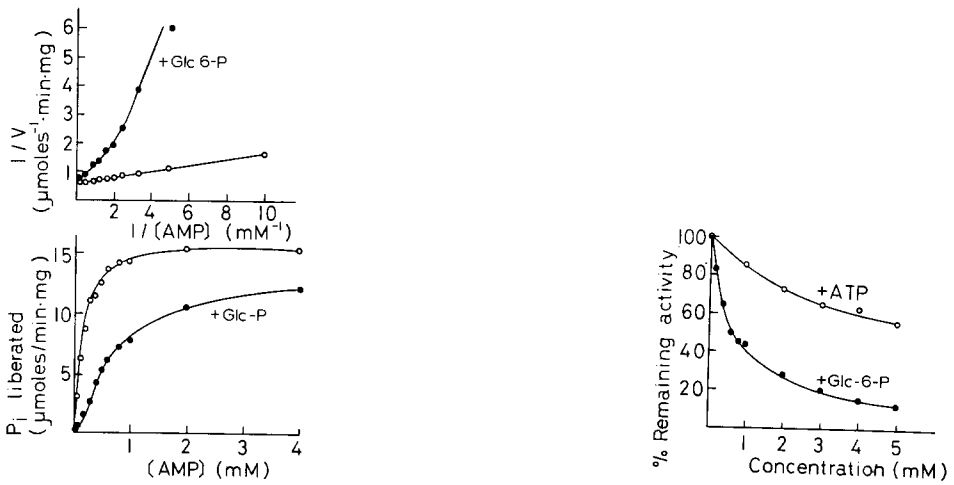


Fig. 8. Effect of AMP concentration on the activity of inactive spleen phosphorylase and suppressing effect of glucose-6-*P*. Enzyme (14 μg) was incubated for 10 min at 30° in 0.02 M glycerophosphate buffer (pH 6.7) containing 16 mM glucose-1-*P*, 0.5% glycogen, 15 mM cysteine and various concentrations of AMP with or without 0.5 mM glucose-6-*P*.

Fig. 9. Effect of glucose-6-*P* and ATP concentration on the AMP activation of inactive spleen phosphorylase. Enzyme (14 μg) was incubated for 10 min at 30° in 0.02 M glycerophosphate buffer (pH 6.7) containing 2 mM AMP, 15 mM cysteine, 16 mM glucose-1-*P*, 0.5% glycogen and various concentrations of glucose-6-*P* or ATP.

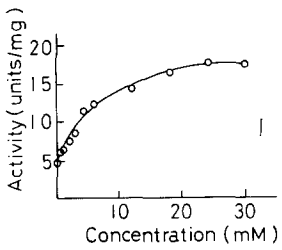


Fig. 10. Effect of cysteine concentration on the activity of inactive spleen phosphorylase. Enzyme (17 μg) was incubated for 10 min at 30° in 0.02 M glycerophosphate buffer (pH 6.7) containing 16 mM glucose-1-*P*, 0.5% glycogen, 2 mM AMP and various concentrations of cysteine as indicated.

spleen phosphorylase in the presence of 2 mM AMP as a function of glycogen or glucose-1-*P* concentration are shown in Fig. 7. The apparent K_m for glycogen and glucose-1-*P* were calculated to be 0.016% and 4.8 mM, respectively. In the presence of a saturated concentration of glycogen or soluble starch (both 0.5%), the maximal velocity for soluble starch was 35% of that for glycogen.

Activation by AMP and suppressing effect of glucose-6-P and ATP. The relation between the concentration of AMP and the reaction rate revealed a curve of Michaelis–Menten type giving a K_a of the enzyme for AMP of 0.2 mM. The maximal velocity was attained at 2 mM AMP (Fig. 8). As shown in the same figure, the activation by AMP was competitively inhibited by glucose-6-*P*. An analogous but lesser effect was observed with ATP. In the presence of 0.5 mM glucose-6-*P*, the Lineweaver–Burk plot for AMP was non-linear. The inhibitory actions of glucose-6-*P* and ATP upon the activation by AMP reached approximately 90 and 50%, respectively, under the conditions tested (Fig. 9). These results suggest that glucose-6-*P* and ATP may play a role in the regulation of spleen phosphorylase activity, as observed for the muscle enzyme[37].

Activation by cysteine. Fig. 10 shows the effect of cysteine on the activity of spleen inactive phosphorylase. The enzymic activity increased with the concentration of cysteine added, and about a 3-fold increase in the activity was noted in the presence of 24 mM cysteine. Cysteine is believed to activate the reaction and not to reactivate the enzyme, since preincubation of the enzyme with the reagent followed by the assay of enzymic activity with the diluted solution resulted in no increase in activity. Although a similar activating effect of cysteine has been observed in muscle phosphorylase *b* (ref. 38), the liver enzyme was not activated by this reagent[2].

Effect of salts. Fig. 11 shows the effect of salts on the activity of spleen inactive phosphorylase. As with phosphorylase from liver[32], corpus luteum[6] and chloroma[10], it was activated by Na_2SO_4 when the activities were assayed in the presence of 2 mM AMP. The addition of 0.7 M Na_2SO_4 enhanced the activity up to a 3-fold increase. However, Na_2SO_4 could not substitute for AMP as an activator. $(NH_4)_2SO_4$ was not effective.

Although inactive spleen phosphorylase was scarcely activated by NaF, as was muscle phosphorylase *b* (refs. 39, 40), when assayed in the presence of 2 mM AMP, a considerable activation by this salt was observed when it was assayed in the presence of a low concentration of AMP. As shown in Fig. 12, a Lineweaver–Burk plot

for AMP below 2 mM was non-linear in the absence of NaF. However, it gave a straight line, on the addition of 0.2 M NaF, with an apparent K_a value for AMP of 0.03 mM. Neither NaF nor Na_2SO_4 could substitute for AMP. $NaClO_4$ exhibited an inhibitory effect on inactive spleen phosphorylase. In the presence of 0.2 M $NaClO_4$, the activity was almost completely lost, as observed for the muscle enzyme[39,40]. It could, however, be recovered by removal of the salt.

Conversion to the active form

Inactive spleen phosphorylase was incubated with 0.02 M ATP, 0.02 M $MgCl_2$ and the crude preparation of phosphorylase kinase at pH 7.8 and 30°. As shown in Fig. 13, the activities both with and without AMP increased up to the maximum

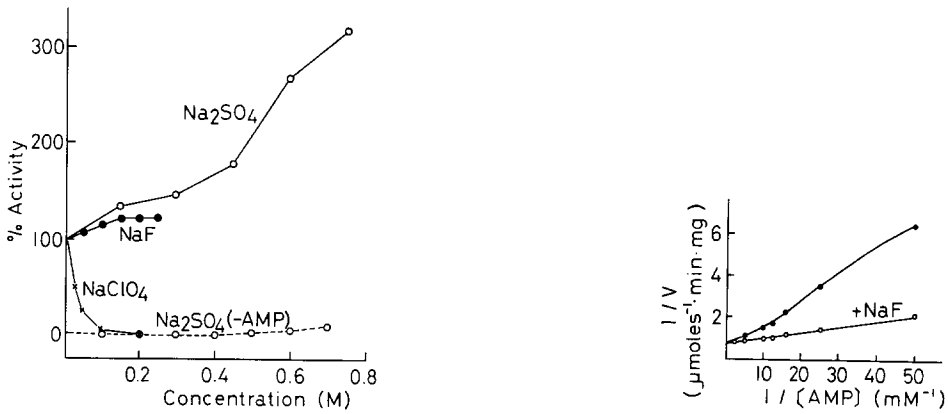
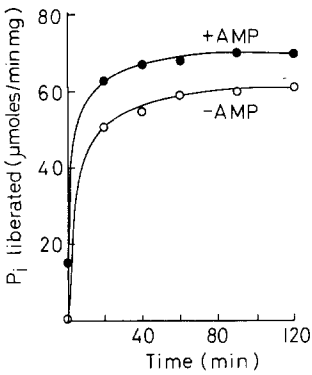


Fig. 11. Effect of salt concentration on the activity of inactive spleen phosphorylase. Enzyme (12 μg) was incubated for 10 min at 30° in 0.02 M glycerophosphate buffer (pH 6.7) containing 16 mM glucose-1-*P*, 0.5% glycogen, 15 mM cysteine, 2 mM AMP and various concentrations of salts as indicated. The final pH was adjusted to 6.7.

Fig. 12. Effect of NaF concentration on the activity of inactive spleen phosphorylase at low concentrations of AMP. The reaction mixture (1 ml) contained 0.02 M glycerophosphate buffer (pH 6.7) 16 mM glucose-1-*P*, 0.5% glycogen, 15 mM cysteine, 37 μg of enzyme and various concentrations of AMP in the presence or absence of 0.2 M NaF. The final pH was adjusted to 6.7. Incubation was carried out at 30° for 10 min.

within 60 min. Omitting ATP or phosphorylase kinase from the system yielded only a slight increase of phosphorylase activity. Thus, the activation observed was regarded as the conversion of inactive phosphorylase to the active form. Without AMP, the activity of the active form corresponded to approximately 4 times that of the inactive form with AMP. This behavior of spleen phosphorylase is comparable to that with the kidney enzyme[20] and shows intermediate properties between muscle and liver phosphorylase.

Fig. 14 shows the elution pattern of a mixture of active and inactive spleen phosphorylase from Sephadex G-200 column chromatography. Although a small peak, which might be aggregated forms of the enzyme, appeared and a considerable amount of the activity was lost during the process, major portions of the remaining activities with and without AMP were eluted in exactly the same fractions. This indicates that the molecular weights of the active and inactive forms of spleen phosphorylase are



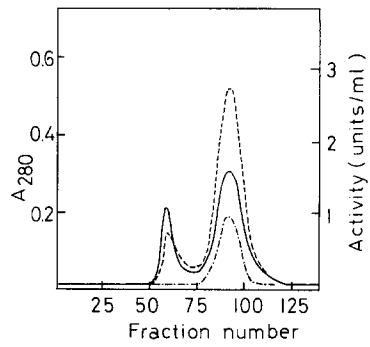


Fig. 13. Conversion of inactive spleen phosphorylase to the active form. Purified inactive phosphorylase (76 μg) was incubated at 30° in a conversion system, which contained 25 mM Tris–HCl buffer (pH 7.8), 10 mM ATP, 20 mM $MgCl_2$, 50 mM NaF and the 20–40% saturated $(NH_4)_2SO_4$ fraction of the crude extract from bovine spleen (1.7 mg protein), in a total volume of 0.2 ml. 10 μl aliquots were taken at various time intervals and transferred to a standard assay system containing 20 mM EDTA and 50 mM NaF, with or without 2 mM AMP. Activity was determined after incubation at 30° for 10 min, and corrected for small blank values by omitting inactive phosphorylase or ATP.

Fig. 14. Elution pattern of a mixture of active and inactive spleen phosphorylases from Sephadex G-200 column chromatography. Active spleen phosphorylase was prepared as in MATERIALS AND METHODS, mixed with the inactive enzyme (5 and 7 mg, respectively), and chromatographed on a Sephadex G-200 column (2.5 cm × 100 cm) that had been equilibrated with 0.01 M Tris–HCl buffer (pH 7.4) containing 2 mM mercaptoethanol and 0.1 M NaF. Protein was eluted with the same buffer. Fractions of 3 ml were collected and assayed for absorbance at 280 nm and activity with or without 2 mM AMP. ———, absorbance at 280 nm; - - - - -, activity with AMP, —·—, activity without AMP.

similar, and that no dimerization occurs upon the conversion. Similar conclusions were drawn with liver[4] and uterine[19] phosphorylase.

DISCUSSION

We have previously demonstrated that potato phosphorylase and rabbit muscle phosphorylase *b* resemble each other in their molecular properties in spite of differing in their mechanisms of controlling the enzymic activity[27,33,41–43]. In connection with these comparative studies, some of the present results on spleen phosphorylase are compared with those of skeletal muscle phosphorylase *b* (ref. 31) and liver inactive phosphorylase[32] (Table III). Similarities between the enzyme from these three tissues are noticed in their molecular properties: molecular weight, amino acid composition, content of pyridoxal-5′-*P* and sedimentation coefficient. However, their catalytic properties differ: the spleen enzyme resembles muscle phosphorylase *b* in the activation by cysteine and NaF and inactivation by $NaClO_4$. Spleen inactive phosphorylase was activated by Na_2SO_4 as was the liver inactive enzyme. The activating effect of AMP on spleen inactive phosphorylase is an intermediate property of the muscle and liver enzyme. Whereas muscle phosphorylase *b* underwent a doubling of molecular weight during enzymic conversion to the active form, the inactive liver and spleen enzyme did not.

Therefore, spleen phosphorylase appears to be a type intermediate between

TABLE III

A COMPARISON OF THE PROPERTIES OF PHOSPHORYLASES FROM MUSCLE, SPLEEN AND LIVER

Property	*Muscle**	*Spleen***	*Liver****
Activation by AMP	++	+	−
Activation by Na_2SO_4	−	+	++
Activation by cysteine	+	+	−
Inhibition by SH-reagent	+	±	+
Optimal pH	6.7	6.7	6.6
Pyridoxal-5′-*P* (moles/100 000 g)	0.95	1.0	1.2
$s_{20,w}$	8.2	8.5	8.4
Molecular weight change on enzymic activation	Double	No change	No change

* Rabbit muscle phosphorylase *b* (ref. 31).
** Inactive bovine spleen phosphorylase (the present study).
*** Inactive pig liver phosphorylase[32].

muscle and liver phosphorylase. VILLAR-PALASI AND GAZQUEZ-MARTINEZ[20] reached a similar conclusion on kidney phosphorylase on the basis of the activation by AMP, although its molecular properties were not studied. It is assumed that spleen and kidney phosphorylase are similar and classified in the third group in mammalian phosphorylases in addition to the two groups represented by skeletal muscle and liver phosphorylases, respectively.

REFERENCES

1 G. T. CORI, S. P. COLOWICK AND C. F. CORI, *J. Biol. Chem.*, 123 (1938) 381.
2 E. W. SUTHERLAND AND W. D. WOSILAIT, *J. Biol. Chem.*, 218 (1956) 459.
3 P. J. KELLER AND G. T. CORI, *Biochim. Biophys. Acta*, 12 (1953) 235.
4 W. D. WOSILAIT AND E. W. SUTHERLAND, *J. Biol. Chem.*, 218 (1956) 469.
5 G. A. RILEY AND R. C. HAYNES, JR., *J. Biol. Chem.*, 238 (1963) 1563.
6 A. A. YUNIS AND S. A. ASSAF, *Biochemistry*, 9 (1970) 4381.
7 W. C. HÜLSMANN, T. L. OEI AND S. V. CREVELD, *Lancet*, 9 (1961) 581.
8 H. E. WILLIAMS AND J. B. FIELD, *J. Clin. Invest.*, 40 (1961) 1841.
9 A. A. YUNIS AND G. K. ARIMURA, *Cancer Res.*, 24 (1964) 489.
10 A. A. YUNIS AND G. K. ARIMURA, *Biochim. Biophys. Acta*, 118 (1966) 325.
11 A. A. YUNIS AND G. K. ARIMURA, *Biochim. Biophys. Acta*, 118 (1966) 335.
12 C. H. DAVIS, L. H. SCHLISELFELD, D. P. WOLF, C. A. LEAVITT AND E. G. KREBS, *J. Biol. Chem.*, 242 (1967) 4824.
13 K. HANABUSA AND H. KOHNO, *J. Biochem.*, 66 (1969) 69.
14 E. MOHME-LUNDHOLM, *Acta Physiol. Scand.*, 59 (1963) 74.
15 G. T. CORI AND C. F. CORI, *J. Biol. Chem.*, 135 (1940) 733.
16 J. BELFORD AND M. R. FEINLIEB, *Biochem. Pharmacol.*, 6 (1961) 189.
17 B. M. BRECKENRIDGE AND J. H. NORMAN, *J. Neurochem.*, 9 (1962) 383.
18 O. H. LOWRY, D. W. SCHULZ AND J. V. PASSONNEAU, *J. Biol. Chem.*, 242 (1967) 271.
19 H. P. SCHANE, *Anal. Biochem.*, 11 (1965) 371.
20 C. VILLAR-PALASI AND I. GAZQUEZ-MARTINEZ, *Biochim. Biophys. Acta*, 159 (1968) 479.
21 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
22 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
23 L. ORNSTEIN AND H. J. DAVIS, *Disc electrophoresis*, Distillation Products Industries, Rochester, N. Y., 1963.
24 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
25 H. WADA AND E. E. SNELL, *J. Biol. Chem.*, 236 (1961) 2089.
26 S. MOORE AND W. H. STEIN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology* Vol. 6, Academic Press, New York, 1963, p. 819.

27 A. KAMOGAWA, T. FUKUI AND Z. NIKUNI, *J. Biochem.*, 63 (1968) 361.
28 K. HANABUSA, T. KOHNO, S. ADACHI AND H. KOBAYASHI, *J. Biochem.*, 62 (1967) 194.
29 V. L. SEERY, E. H. FISCHER AND D. C. TELLER, *Biochemistry*, 6 (1967) 3315.
30 D. P. WOLF, E. H. FISCHER AND E. G. KREBS, *Biochemistry*, 9 (1970) 1923.
31 A. B. KENT, E. G. KREBS AND E. H. FISCHER, *J. Biol. Chem.*, 232 (1958) 549.
32 M. M. APPLEMANN, E. G. KREBS AND E. H. FISCHER, *Biochemistry*, 5 (1966) 2101.
33 A. KAMOGAWA, *Biochim. Biophys. Acta*, 178 (1969) 459.
34 C. L. SEVILLA AND E. H. FISCHER, *Biochemistry*, 8 (1969) 2161.
35 G. L. ELLMAN, *Arch. Biochem. Biophys.*, 82 (1959) 70.
36 K. KLEPPE AND S. DAMJANOVICH, *Biochim. Biophys. Acta*, 185 (1969) 88.
37 H. E. MORGAN AND A. PARMEGGIANI, *J. Biol. Chem.*, 239 (1964) 2440.
38 C. F. CORI, G. T. CORI AND A. A. GREEN, *J. Biol. Chem.*, 151 (1943) 39.
39 R. W. SEALOCK AND D. J. GRAVES, *Biochemistry*, 6 (1967) 201.
40 D. J. GRAVES, S. A. S. MANN, G. PHILIP AND R. J. OLIVERIA, *J. Biol. Chem.*, 243 (1968) 6090.
41 A. KAMOGAWA, T. FUKUI AND Z. NIKUNI, *J. Biochem.*, 64 (1968) 709.
42 T. FUKUI, A. KAMOGAWA AND Z. NIKUNI, *J. Biochem.*, 67 (1970) 211.
43 A. KAMOGAWA, T. FUKUI AND Z. NIKUNI, *Agr. Biol. Chem.*, 35 (1971) 249.