Eur. J. Biochem. 74, 233–241 (1977)

Antibodies and Autoantibodies of Glycogen Phosphorylase *b*: Inactivation of Pig and Rabbit Enzymes

Socrates J. TZARTOS and Athanasios E. EVANGELOPOULOS

Department of Biochemistry, The National Hellenic Research Foundation, Athens

(Received September 28, 1976)

Pig skeletal muscle glycogen phosphorylase *b* was purified using ammonium sulfate fractionation, DEAE-Sephadex A-50 and Sephadex G-200 column chromatography. The purified enzyme was used to immunize rabbits in the presence or in the absence of complete Freund adjuvant.

Antibodies against pig phosphorylase in pure form were isolated from rabbit antisera using insoluble immunoadsorbents of pig phosphorylase. Autoantibodies against the rabbit enzyme were obtained from the same antisera using insoluble immunoadsorbents of rabbit phosphorylase. Complete inactivation of pig phosphorylase was accomplished by an antibody/enzyme molar ratio equal to 4 and autoantibody/enzyme molar ratio equal to 130. Complete inactivation of rabbit phosphorylase was accomplished by an antibody/enzyme molar ratio equal to 250 and autoantibody/enzyme molar ratio equal to 160. Passive haemagglutination technique gave positive results with minimum amounts of 0.02 μg/ml and 0.8 μg/ml for pig and rabbit phosphorylase respectively.

Kinetic experiments have shown that antibodies and autoantibodies act as noncompetitive inhibitors of both enzymes with respect to AMP and glucose 1-phosphate but exhibit a mixed type of inhibition with respect to glycogen. When glycogen hydrolysates were used as substrate in place of intact glycogen molecules a pronounced decrease in the inhibitory capacity of antienzyme on the enzyme was demonstrated.

Enzymes present some advantages for immunological studies since they are equipped with one more parameter compared to other proteins, their catalytic activity. Inhibition of the enzymatic activity because of enzyme-antienzyme interaction is usually due to steric hindrance or to conformational changes induced on the enzyme [1,2]. Mammalian skeletal muscle glycogen phosphorylase exists in two forms, that have been designated as *a* and *b*. Pyridoxal phosphate, the coenzyme of both forms, is indispensable for their catalytic activity. Activation of phosphorylase *b* in contrast to *a* has an absolute requirement for AMP [3,4]. Extensive immunological work [5–9] has been done on this enzyme since Henion and Sutherland [10] studied the differences of phosphorylase among different species using immunological methods.

The ability of antibody molecules against a particular enzyme, to cross-react (act as autoantibodies) and inactivate [5,6] the respective enzyme of the immunized animal, might have some biological and immunochemical significance. In the present report we have studied the autoantibody capacity of antiphosphorylase preparations isolated through insoluble immunoadsorbents from antisera of rabbits immunized with pig muscle glycogen phosphorylase *b*. The distribution of the autoantibody capacity in the heterogeneous antiphosphorylase mixture, the conditions of their biosynthesis and the kinetics of the systems pig or rabbit enzyme with antibodies or autoantibodies were also studied.

Supported by Grants from the National Hellenic Research Foundation and World Health Organization.

Enzyme. Glycogen phosphorylase or 1,4-α-D-glucan:orthophosphate α-glucosyltransferase (EC 2.4.1.1).

EXPERIMENTAL PROCEDURE

Materials

Glycogen phosphorylase *b* was isolated from rabbit skeletal muscles according to the procedure of Fischer and Krebs [11]. The enzyme was recrystallized at least three times at 0 °C from a buffer of pH 6.8 consisting of 0.05 M glycerol-2-*P*, 0.05 M 2-mercaptoethanol, 1.0 mM AMP and 0.01 M Mg^{2+}. No addi-

tional AMP or Mg^{2+} was added after the first recrystallization. Glycogen phosphorylase *b* from the cock muscle was prepared by the method of Jokay and Karczag [12] while the same enzyme from pig skeletal muscle was purified by a method described in a following section.

Glucose-1-*P* and AMP were purchased from Fluca. Glycerol-2-*P*, cysteine, glycine and EDTA were obtained from BDH. Glycogen oyster was a product of Koch-Light Laboratories while bovine serum albumin and 2-mercaptoethanol were products of Sigma.

Methods

Rabbit antisera against pig muscle phosphorylase *b* were prepared according to the following procedure. One-year-old New Zealand rabbits received for 4 consecutive weeks, at weekly intervals, 4 mg of pig muscle phosphorylase *b*. For the first two weeks the antigen was injected subcutaneously in many sites at the back of the animals as emulsions with complete Freund adjuvant. For the remaining two weeks the antigens were introduced as suspensions in calcium phosphate gels. Fifty days after the last injection, rabbits received two booster subcutaneous injections, at an interval of one week, of 4 mg of the same enzyme *in* buffered saline solution., Five days after each injection animals were bled from the ear marginal vein. The antisera were separated by the usual procedure and stored at − 20 °C.

Phosphorylase activity was determined in the direction of glycogen synthesis at 30 °C using the procedure of Hedrick and Fischer [13] in presence of 1.0 mM AMP and 0.016 M glucose-1-*P* for rabbit phosphorylase or in presence of 1.0 mM AMP and 0.070 M glucose-1-*P* for pig phosphorylase. One unit of activity is that amount of enzyme releasing 1 μmol of P_i from glucose-1-*P* per min at 30 °C.

Phosphorylase inactivation by antisera or purified antibodies was accomplished by incubating 4 μg of pig or 1 μg of rabbit phosphorylase with the proper amount of inhibitor in a final volume of 0.2 ml. After 15 or 60 min incubation at 30 °C for pig or rabbit enzyme respectively, aliquots of 0.1 ml were removed and transferred to the substrate mixture for determination of the residual enzymatic activity. In cases where protein concentration of the samples was high, for instance when antisera were used in the place of purified antibody the reaction was stopped by adding 5% trichloroacetic acid followed by centrifugation. The liberated inorganic phosphate in the supernatant was estimated colorimetrically by the method of Fiske and Subbarow [14]. Parallel experiments in the absence of any protein or in the presence of normal non-immune serum were run for control. Enzyme concentration was determined spectrophotometrically at 280 nm using a value for $A_{280}^{1\%}$ of 13.2 [15]. Protein concentration of immunoglobulin fraction or of purified antibodies was determined either by the method of Lowry *et al.* [16] or by absorption at 280 nm using rabbit immunoglobulin as standard. The molecular weight of the dimeric enzyme was taken as 200000 [17] while the average molecular weights for antibodies was assumed equal to 150000. Absorption spectra were taken by means of a Perkin-Elmer 356 Double Beam UV-Visible recording spectrophotometer.

Acrylamide disc electrophoresis was performed on a Sandon instrument as described previously [18]. Precipitin reactions were carried out in capillary tubes [19], quantitative immunoprecipitation [19] and Ouchterlony diffusion technique [20]. Passive haemaglutination technique was applied as described by Herberd [21]. Ultracentrifugation studies were performed in a MSE Centriscan 75 preparative and analytical ultracentrifuge.

Preparation of immunoadsorbent was made by the procedures of Avrameas and Ternynck [22] and Patramani *et al.* [23]. Insolubilization of enzyme-albumin solutions (5 mg phosphorylase + 25 mg bovine serum albumin per ml) by glutaraldehyde (60 mM) takes place easily in 0.1 M Tris buffer pH 7.2, with difficulty in 0.1 M phosphate buffer pH 6.4 while in 0.1 M carbonate pH 8.8 no precipitate of enzyme-albumin was observed. Pig phosphorylase polymerizes more easily in absence than in presence of albumin at pH 7.2 (30 mg/ml protein). However, immunoadsorbents prepared through copolymerization of phosphorylase and albumin absorb higher quantities of antibodies than insolubilized enzyme alone (Table 1). For immunoadsorbance, 30 ml of rabbit antiphosphorylase antiserum were mixed with 600 mg of immunoadsorbent (insolubilized 100 mg pig phosphorylase + 500 mg albumin in 60 mM glutaraldehyde/0.1 M Tris) for 60 min. After the removal of non-adsorbed proteins from the complex immunoadsorbent − antibodies by extensive washing and centrifugation, dissociation of the adsorbed antibodies was accomplished by suspending the complex in 6 ml 0.2 M glycine-HCl buffer pH 2.7 for 45 min at 20 °C. The treatment with glycine-HCl buffer was repeated twice. The supernatants were pooled, adjusted to neutral pH with NaOH and dialyzed in the cold room against 20 mM Tris/2 mM EDTA/0.85% NaCl buffer pH 6.8. The dialysed solution of antibodies was subsequently concentrated by ultrafiltration.

Glycogen fragmentation was achieved as described previously [24]. Three 2-ml samples (I, II, III) of 6% glycogen in 0.6 N HCl were placed in a boiling water bath for 1, 2, and 5 min respectively. At the end of each time samples were neutralized by adding the proper amount of NaOH. A fourth sample was used as a blank. The observed activity of pig and rabbit

Table 1. *Cross-link of phosphorylase alone or with albumin after the addition of glutaraldehyde (60 mM) at various pH values*
The first three preparations consisted of 15 mg pig phosphorylase plus 75 mg albumin (in 3 ml). The fourth preparation contained only 15 mg of pig enzyme (in 0.5 ml)

Expt number	pH	Time of precipitate formation	Non-precipitated protein	Solubility in 0.2 N		Remaining inhibitory capacity of adsorbed antisera
				NaOH	HCl	
		min	%			%
1	6.4	50	29	–	–	55
2	7.2	20	18	–	–	35
3	8.8	∞	100			
4	7.2	2	6.6	–	–	70

Table 2. *Purification scheme of pig skeletal muscle phosphorylase* b

Fraction	Volume	Protein	Total activity	Specific activity	Recovery	Purification
	ml	mg	U	U/mg	%	-fold
Supernatant of 2 kg homogenized skeletal muscle	4100	42000	16800	0.4	100	1
35% ammonium sulfate pellet	50	950	12200	12.8	73	32
DEAE-Sephadex chromatography	19	315	6200	19.5	37	49
Sephadex G-200 chromatography	14.8	222	5020	22.6	30	56

phosphorylases, when intact glycogen molecules were substituted by these fragments was 86, 18, 12%, and 81, 15, 13% for pig and rabbit enzymes respectively.

RESULTS

PURIFICATION AND PROPERTIES OF PIG SKELETAL MUSCLE PHOSPHORYLASE *b*

Pig skeletal muscle phosphorylase *b* was isolated using DEAE A-50 and G-200 Sephadex chromatography since our attempts to isolate the enzyme through crystallization according to a previous procedure were unsuccessful [25].

Initial Steps

Muscle homogenization, acid precipitation at pH 5.2 and ammonium sulfate precipitation at 4 °C were performed as described previously for pig and rabbit phosphorylases [25, 11]. Protein precipitation was accomplished using 35% ammonium sulfate saturation instead of 45% [25] since preliminary experiments showed that the former saturation gave enzyme preparation of a higher purity.

DEAE-Sephadex A-50 Chromatography

The clear supernatant containing 950 mg protein (50 ml, 19 mg/ml) obtained from the previous steps was applied on the top of a DEAE-Sephadex A-50 column (3.5 × 52 cm) equilibrated with the initial buffer used for dialysis (3 mM cysteine/1.0 mM EDTA/4 mM glycerol-2-*P* pH 7.0). Elution of the column was performed with 500 ml of the initial buffer followed by a linear gradient formed by 600 ml of the initial buffer and 600 ml of 3 mM cysteine/1 mM EDTA/60 mM glycerol-2-*P* buffer. Pig phosphorylase was eluted from the column as a sharp peak centered at about 1.100 ml of the total buffer used in the elution diagram of the column. No enzymatic activity was detected in any of the fractions of the DEAE-Sephadex A-50 column in absence of AMP, the degree of enzyme purification accomplished by this column is shown in the Table 2.

Sephadex G-200 Chromatography

The fractions with the highest phosphorylase activity from the previous step were pooled and concentrated by ultrafiltration to 20 ml (about 300 mg of protein). The concentrated solution was applied on the top of a Sephadex G-200 column (2.5 × 80 cm)

equilibrated with a buffer containing 3 mM cysteine/1 mM EDTA/50 mM glycerol-2-*P* pH 7.0. The same buffer was used for the elution of the column. The enzyme was eluted from the column as a sharp peak centered at 220 ml of the volume of the elution diagram. Fractions of the highest phosphorylase activity from the Sephadex G-200 column were pooled and concentrated by ultrafiltration to 15 ml (about 220 mg of enzyme). In Table 2 the summary of the purification procedure for a typical enzyme preparation is given. All efforts to crystallize the isolated pig skeletal muscle glycogen phosphorylase *b* were unsuccessful.

Properties of Pig Phosphorylase b

Disc acrylamide gel electrophoresis of pig phosphorylase *b* obtained in the above manner migrates as a single band at pH 8.0, similar in appearance but slightly more mobile than rabbit phosphorylase *b*.

The absorption spectrum of the purified pig skeletal muscle glycogen phosphorylase *b* obtained by means of a recording spectrophotometer exhibited similar absorption maxima and minima with those of the spectrum of the rabbit enzyme [26]. The spectrum between 230 and 500 nm for two enzyme concentrations is presented in Fig. 1 since it was not reported previously [25].

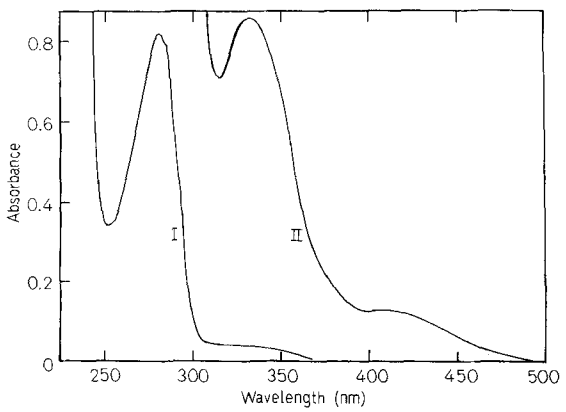

Fig. 1. *Absorption spectrum of pig phosphorylase.* Buffer was 1.0 mM EDTA/50 mM glycerol-*P* pH 7.0. (I) 0.6 mg/ml enzyme; (II) 13 mg/ml enzyme

In order to obtain information concerning the molecular weight of the pig enzyme comparative studies were performed using 20 mg of each pig and rabbit phosphorylase *b* and two identical Sephadex G-200 columns (2 × 72 cm). It was shown that the elution of the two enzymes was taking place in the same volume of the elution diagrams. Sedimentation patterns of pig and rabbit phosphorylases were obtained using an analytical ultracentrifuge. The two enzymes were subjected to ultracentrifugation in 0.05 M glycerol-2-*P* pH 6.8 (4 mg/ml, 20 ± 1 °C), at a rotor speed of 59750 rev./min. In both cases a single peak was obtained in the Schlieren patterns. The calculated sedimentation coefficients $s_{20,w}$ were found equal to 8.7 and 8.5 for pig and rabbit phosphorylase *b* respectively. The results of the previous experiments suggest that the two enzymes have very similar molecular weights (Table 3).

Comparative kinetic studies of pig and rabbit enzymes were also performed. In Table 3 are shown the obtained Michaelis constants (K_m) and maximum velocities (V) for AMP, glucose-1-*P* and glycogen. Although the V values determined for substrates and activator were similar for both enzymes, the obtained numbers concerning the K_m for AMP and glucose-1-*P* were more than three-fold higher for pig than for rabbit phosphorylase.

STUDIES WITH ANTIPHOSPHORYLASE

Reactivity of Antisera

Interaction of pig and rabbit phosphorylase with rabbit antiserum to pig phosphorylase was studied by precipitin tests and enzymatic inhibition. Ouchterlony diffusion plates were prepared using pure pig and rabbit phosphorylase at concentrations ranging from 0.5–10 mg/ml and undiluted sera. In all cases with pig phosphorylase single precipitin lines were obtained while with rabbit phosphorylase no precipitin line was observed. Quantitative precipitin reactions were carried out with a constant amount of undiluted antiserum (0.5 ml) and increasing amounts (10–500 µg) of pig and rabbit enzymes. Precipitation was observed from 20 µg of pig phosphorylase upwards and the

Table 3. *Kinetic parameters and constants of pig and rabbit phosphorylase* b
For AMP, glycogen was 1% and glucose-1-*P* was 70 mM for pig and 16 mM for rabbit enzyme. For glucose-1-*P*, glycogen was 1% and AMP was 2 mM. For glycogen, AMP was 2 mM and glucose-1-*P* was 70 mM for pig and 16 mM for rabbit phosphorylase

Phosphorylase	AMP		Glucose-1-*P*		Glycogen		$s_{20,w}$	Elution volume Sephadex G-200 (2 × 72 cm)
	K_m	V	K_m	V	K_m	V		
	µM	mg × min^{-1} × (µmol P_i)$^{-1}$	mM	mg × min^{-1} × (µmol P_i)$^{-1}$	%	mg × min^{-1} × (µmol P_i)$^{-1}$	S	
Pig	220	50	31.7	83	0.041	45.5	8.7	135–145
Rabbit	67	43.5	7	82	0.036	51	8.5	135–145

zone of equivalence for the enzyme was about 120 μg. No precipitate was observed with rabbit phosphorylase.

Total inactivation of pig phosphorylase was accomplished by antisera of all immunized rabbits. It is of interest that the same antisera when used in high quantities were able to inhibit also rabbit phosphorylase. The effect of a representative rabbit antiserum on pig and rabbit phosphorylase is shown in Fig. 2. In the same figure is given the inactivation of the rabbit phosphorylase isolated from the same immunized animal which was used to obtain the antiserum. The rabbit was sacrificed two months after the last injection of antigen. No differences between the two rabbit enzymes were observed. The inhibition of pig and rabbit phosphorylases by antisera of three immunized rabbits during the process of immnization is shown in Fig. 3. It appears that the enzymatic property of the employed antigen reveals an interesting autoantibody activity of rabbit antisera to pig phosphorylase *b* worthy of further study.

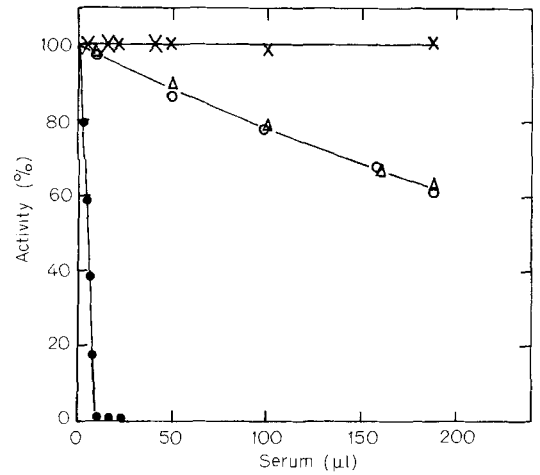

Fig. 2. *Inhibition of pig and rabbit phosphorylase by a rabbit antiserum to pig phosphorylase.* Effect of antiserum on pig (●), rabbit (○) or the donor of the antiserum rabbit phosphorylase (△). Effect of normal rabbit serum on both enzymes (X, x). Experimental conditions as described in Methods

Properties of Antiphosphorylase

The inactivating capacity of various preparations of purified antiphosphorylase was studied with pig and rabbit phosphorylases. In the Fig. 4A is shown the inhibition of the enzymatic activity of pig phosphorylase *b* by three antiphosphorylase preparations. Complete inactivation of the enzyme is accomplished at an antibody/enzyme molar ratio about 4. At the latter molar ratio of antienzyme/enzyme the effect on rabbit phosphorylase was rather small. However, in higher molar ratios a substantial inactivation of rabbit phosphorylase by pig antiphosphorylase was observed reaching complete inactivation of the rabbit enzyme at a molar ratio of pig antienzyme/rabbit phosphorylase around 200 (Fig. 4B). For purposes of comparison the inactivating capacity of pig antienzyme toward a glycogen phosphorylase of non-mammalian species was tested. Complete inactivation of cock glycogen phosphorylase *b* from skeletal muscles was achieved at a molar ratio antienzyme/enzyme about 25 (Fig. 4B).

Quantitative precipitin tests between pig or rabbit phosphorylases and various concentrations of pig antienzyme are shown in Fig. 5. In contrast to the nega-

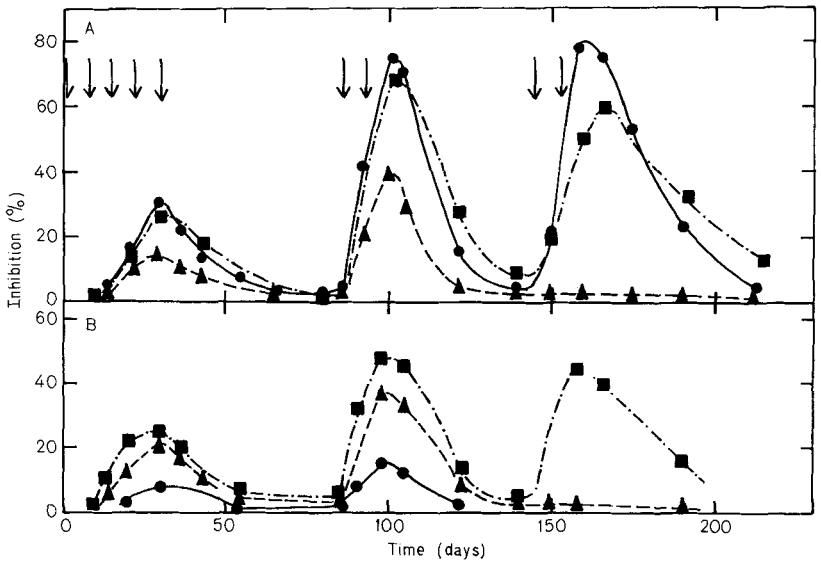

Fig. 3. *Changes in pig (A) and rabbit (B) phosphorylase inhibition by the antisera of three rabbits during the process of immunization.* Antibody-antigen reaction mixtures consisted of: (A) 10 μl antiserum with 4 μg of pig phosphorylase: (B) 160 μl antiserum with 1 μg of rabbit phosphorylase. (↓) Day of the antigen injection. (●, ■, ▲) Antisera of first, second and third rabbit. Rabbit instead of pig phosphorylase was introduced in the last two injections of the third rabbit

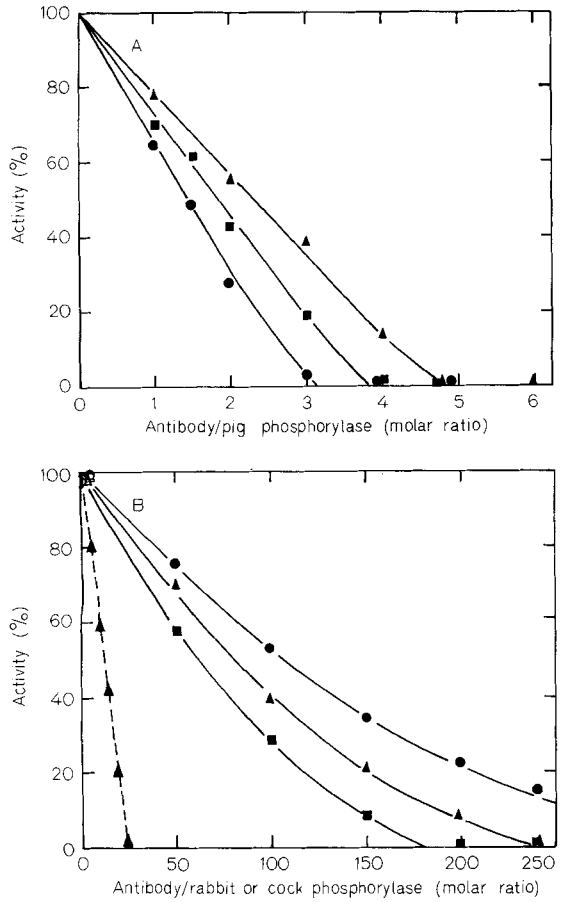

Fig. 4. *Inactivation of pig, rabbit and cock phosphorylases by purified preparations of antienzyme against pig phosphorylase prepared from antisera of three rabbits.* Experimental conditions as described in Methods. (A) Pig; (B) rabbit (—), and cock (----)

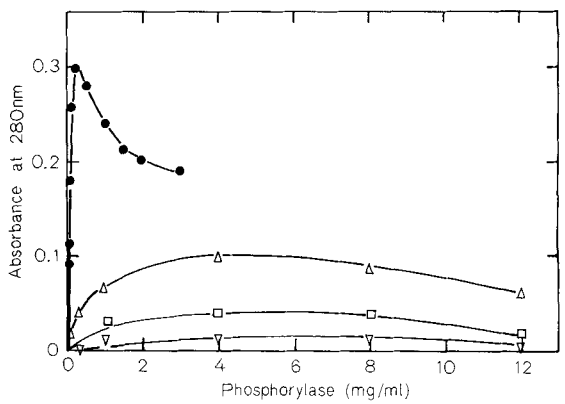

Fig. 5. *Quantitative precipitin reactions of pig (●) and rabbit phosphorylases (▽, □, △) with purified antiphosphorylase.* Increasing amounts of enzymes were added to 1-ml volumes containing 0.5 mg (●), 1 mg (▽), 2 mg (□), and 4 mg (△) of antibody in 0.05 M glycerol-2-*P*/0.85% NaCl pH 7.0 buffer

tive results obtained with antisera, rabbit phosphorylase is able to interact with purified antibodies against the pig enzyme prepared in rabbits and give immunoprecipitates. When the Ouchterlony technique was used, rabbit phosphorylase produced a weak precipitin line only when high concentrations of the reactants were used (20 mg/ml rabbit enzyme and 5 mg/ml antibody). Because of the weak line observed with rabbit enzyme, it was not possible to characterize the extent of antigenic identity of pig and rabbit phosphorylases. Passive haemagglutination technique gave positive results with minimum amounts of 0.02 μg/ml and 0.8 μg/ml for pig and rabbit phosphorylases respectively.

Studies with Autoantibodies

In order to investigate the role of complete Freund adjuvant in the observed cross-reactivity, three rabbits were immunized with pig phosphorylase in absence of complete Freund adjuvant. In two of them the usual amount of antigen in buffered saline solution was injected intravenously while the third rabbit was immunized by hypodermic injections of antigen in saline solution. The obtained antisera, after the second week of immunization, exhibited a cross-reactivity comparable to that of antisera of rabbits immunized with complete Freund adjuvant. In order to test the hypothesis whether these animals were also able to synthesize antibodies when immunized with rabbit phosphorylase, booster injections [27, 28] with rabbit enzyme were made into two rabbits previously immunized with pig phosphorylase. To one of the rabbits the 'antigen' was introduced with complete Freund adjuvant and to the other in buffered saline. The obtained results are shown in the Fig. 3.

The procedure described in the Methods section for the preparation of immunoadsorbent of pig phosphorylase was used to prepare immunoadsorbent of the rabbit enzyme. The immunoadsorbent was mixed gently with 40 ml of rabbit antiserum for 4 h at room temperature under toluene vapours. Applying the same procedure described for the isolation of antibodies, we were able to isolate 9.4 mg of autoantibodies. As it can be seen in Fig. 6 these antibody molecules are of low affinity for both enzymes. Complete inactivation of pig enzyme was accomplished at a molar ratio equal to 130 while the same result for rabbit enzyme was reached at a molar ratio equal to 160. The antiserum used previously for the isolation of autoantibodies was subsequently treated twice again with new rabbit phosphorylase immunoadsorbents for the isolation of remaining autoantibodies. The isolated autoantibodies during the first, second and third immunoadsorption were 9.4, 7.0, and 4.4 mg respectively. It was found after this treatment that the antiserum maintained most of the initial inhibitory capacity against pig enzyme (about 90%), while it has lost the 90% of its inhibitory capacity

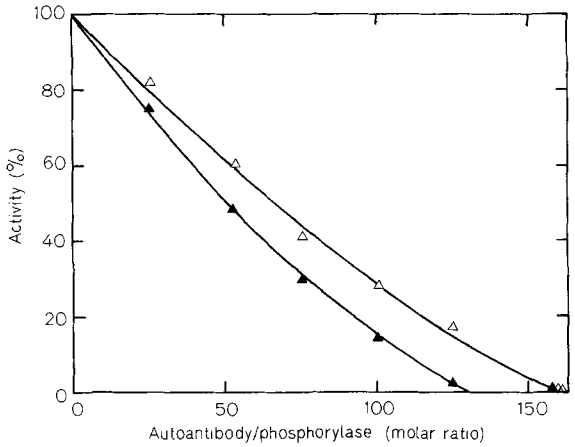

Fig. 6. *Inhibition of pig (▲) and rabbit (△) phosphorylases by autoantibodies.* Experimental conditions as described in Methods

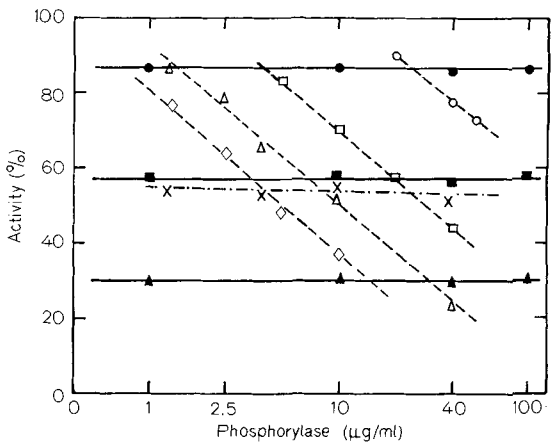

Fig. 7. *Changes in the activity of pig, cock and rabbit phosphorylases as a function of antibody and antigen concentration for constant antibody/antigen molar ratios.* Molar ratios: antibody/pig phosphorylase = 0.5 (●), 1.5 (■), and 2.5 (▲); antibody/cock phosphorylase = 5 (×); antibody/rabbit phosphorylase = 25 (○), 65 (□), 100 (△), and 135 (◇)

for the rabbit enzyme. Autoantibodies isolated during the first immunoadsorption are more competent inhibitors for the rabbit enzyme than the ones isolated in the subsequent steps. The opposite was true for the pig enzyme.

Kinetic Studies

Kinetic experiments performed between phosphorylases and antiphosphorylases in different reaction systems demonstrated that the time required for a given antienzyme quantity to reach its maximum effect on the activity of pig phosphorylase at 30 °C was less than 5 min. The same effect for rabbit phosphorylase needed at least 60 min. It was demonstrated also that for a given antibody/pig enzyme molar ratio, the inhibition remains constant independently of the concentration of the reactants at least from 1 – 100 μg/ml phosphorylase, the same stability was observed with the antibody-cock phosphorylase system. Straight parallel lines were obtained when inhibition was plotted against the logarithm of rabbit enzyme concentration (Fig. 7).

Antibodies and autoantibodies act as noncompetitive inhibitors of pig phosphorylase with respect to AMP and glucose-1-*P* but exhibit a mixed type of inhibition with respect to the macromolecular substrate glycogen (Fig. 8). In order to study further the role of glycogen, partial fragmentation of this substrate was accomplished using mild conditions of hydrolysis by HCl. Enzymatic inhibition studies using antibodies, autoantibodies and glycogen hydrolysates (see Methods section) demonstrated that while 50% inhibition of the pig enzyme is succeded at a molar ratio antienzyme/enzyme around 6, when glycogen hydrolysate II is used as substrate of the enzyme instead of glycogen, the same effect with intact glycogen is reached at a molar ratio around 2. With autoantibodies and pig enzyme 50% inhibition is attained at a molar ratio antienzyme/enzyme about 60 with glycogen as substrate and at more than 250 when glycogen fragments II were used in the system. Glycogen hydrolysate III gave comparable results, while hydrolysate I exhibited similar properties with intact glycogen. In contrast to the behavior of pig phosphorylase no changes in the inhibitory effect of antienzymes on the rabbit enzyme was observed when glycogen substrate was substituted with its hydrolysates. It is worth mentioning that the mixed type inhibition of pig enzyme by antibodies in presence of intact glycogen molecules is converted into non-competitive inhibition when glycogen hydrolysates were used as substrates (Fig. 8C).

DISCUSSION

Pig skeletal muscle glycogen phosphorylase *b* has been isolated according to a new procedure. It was demonstrated that the enzyme has similar molecular weight, absorption spectrum and maximum velocity but higher mobility in acrylamide gel electrophoresis compared to rabbit phosphorylase (*cf.* Fig. 1, Table 3).

The purified antienzyme was able to inactivate not only homologous and heterologous antigens (at a molar ratio antibody/antigen 4 and 25 respectively) but also to cross-react with the respective enzyme of the immunized animal (*cf.* Fig. 4). Autoantibodies isolated from antisera using as immunoadsorbents insoluble rabbit phosphorylase exhibited low affinity for

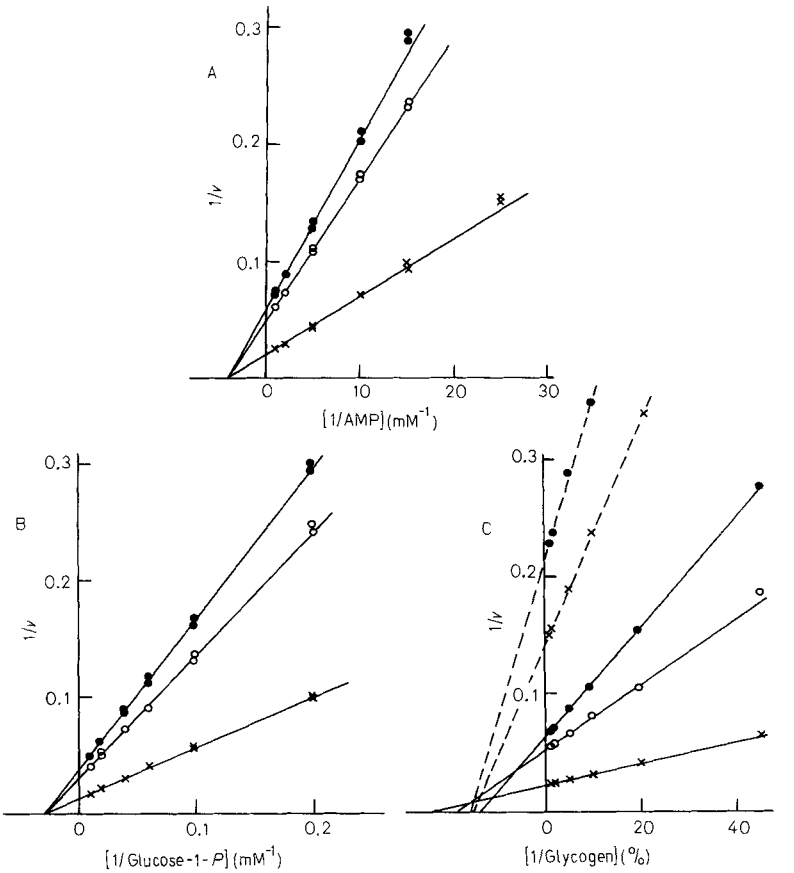

Fig. 8. *Reciprocal plots of pig phosphorylase activity for AMP, glucose-1-*P *and glycogen, in the absence or presence of antibodies and autoantibodies.* Experimental conditions: (A) 70 mM glucose-1-*P*, 1% glycogen: concentration of AMP was varied. (B) 2 mM AMP, 1% glycogen; concentration of glycose-1-*P* was varied. (C) 2 mM AMP, 70 mM glucose-1-*P*; concentration of glycogen (——) and glycogen fragments II (– – –) were varied. No inhibitor (×); antibody/enzyme molar ratio: 2.5 (●); autoantibody/enzyme molar ratio: 70 (○). v is expressed as mg enzyme × min^{-1} × (μmol P_i)$^{-1}$

both enzymes (*cf.* Fig. 6). Complete inactivation of pig and rabbit phosphorylases was accomplished by the isolated autoantibodies at a molar ratio antienzyme/enzyme 130 and 160 respectively. Mechaelides *et al.* [6] using soluble chicken antibody fragments against rabbit phosphorylase *a* had found complete inactivation of rabbit phosphorylase *b* and *a* at a molar ratio antienzyme/enzyme 10 and 120 respectively. Benyamin and Robin had found that lobster muscle kinase is inactivated by its antibody at a molar ratio about 30 [29], while in some enzyme systems as catalase 1, C-1-esterase [30] and trypsin [2], complete inactivation is not attained even at very high antibody/enzyme molar ratios.

Low affinity antibodies (autoantibodies) represent a considerable amount (about 0.5 mg/ml) of the total antibodies in antisera produced during immunization. Their effect on rabbit enzyme inactivation increases with the concentration of the reactants in the system (*cf.* Fig. 7). When rabbits are immunized with enzyme of this same species, they do not produce autoantibodies either in the presence or in the absence of Freund adjuvant. On the other hand, since all immunized animals with pig phosphorylase were able to form autoantibodies, it seems that autoantibody production is unlikely to be due to an abnormality of the lymphoid system of the rabbit. It appears therefore that autoantibodies were produced by the rabbits against the various antigenic determinants of the pig enzyme. This observation has probably a biological significance. It might occur that the three dimensional structure of some of these determinants of the pig enzyme resemble to the conformation of the respective sites of the rabbit enzyme.

A decrease of the inhibitory effect in the system antibodies – pig phosphorylase was observed when glycogen was substituted by glycogen fragments. This is consistent with an inhibition due to steric hindrance. This phenomenon is not observed in the system autoantibodies – rabbit phosphorylase, which could suggest that autoantibodies and antibodies inhibit rabbit and pig phosphorylases through different mechanism.

REFERENCES

1. Cinader, B. (1967) in *Antibodies to Biologically Active Molecules*, (Cinader, B., ed.) pp. 85–137, Pergamon Press, Oxford.
2. Arnon, R. (1975) *Acta Endocrinol. Suppl. 194*, 133–153.

3. Fisher, E. H., Pocker, A. & Saari, J. C. (1970) *Essays Biochem. 6*, 23–68.
4. Graves, D. J. & Wang, J. H. (1972) *The Enzymes, 7*, 435–482.
5. Yunis, A. A. & Krebs, E. C. (1962) *J. Biol. Chem. 237*, 34–39.
6. Michaelidis, M , Sherman, R. & Helmreich, E. (1964) *J. Biol. Chem. 239*, 4171–4181.
7. Michaelides, M. & Helmreich, E. (1966) *Enzymol. Biol. Clin. 7*, 130–143.
8. Jokay, I. & Karczag, E. (1968) *Acta Microb. Acad. Sci. Hung. 15*, 295–306.
9. Jokay, I. & Karczag, E. (1972) *Ann. Immun. Hung. 16*, 199–209.
10. Henion, W. F. & Sutherland, E. W. (1957) *J. Biol. Chem. 224*, 477–488.
11. Fisher, E. H. & Krebs, E. G. (1962) *Methods Enzymol. 5*, 369–373.
12. Jokay, I. & Karczag, E. (1971) *Proc. Microbiol. Res. Group Hung. Acad. Sci. 4*, 125–142.
13. Hedrick, J. L. & Fisher, E. H. (1965) *Biochemistry, 4*, 1337–1343.
14. Fiske, C. H. & SubbaRow, I. (1925) *J. Biol. Chem. 66*, 375–400.
15. Buc, M. H. & Buc, H. (1968) *Symposium on Regulation of Enzymatic Activity and Allosteric Interactions*, Academic Press, New York, N.Y.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
17. Cohen, P., Dueuer, T. & Fisher, E. H. (1971) *Biochemistry, 10*, 2683.
18. Katsiris, K. D., Dimitropoulos, C. G., Patramanis, I. M., Pavlatos, M. P. & Evangelopoulos, A. E. (1972) *Eur. J. Biochem. 27*, 23–30.
19. Kabat, E. & Meyer, M. M. (1964) *Experimental Immunochemistry*, Charles Thomas, Springfield, Illinois.
20. Ouchterlony, O. (1967) in *Handbook of Experimental Immunology* (Weir, D. M., ed.) pp. 655–706, Blackwell, Oxford.
21. Herbert, J. (1967) In *Handbook of Experimental Immunology* (Weir, D. M., ed.) pp. 720–744, Blackwell, Oxford.
22. Avrameas, S. & Ternynck, T. (1969) *Immunochemistry, 6*, 53–66.
23. Patramani, I., Katsiris, K., Pistevou, E., Kalogerakos, T., Pavlatos, M. & Evangelopoulos, A. E. (1969) *Eur. J. Biochem. 11*, 28–36.
24. Clark, J. M., Jr (1964) *Experimental Biochemistry*, Freeman, W. H. and Co., San Francisco.
25. Hanabusa, K., Kanno, T., Adachi, S. & Kobayashi, H. (1967) *J. Biochem. 62*, 194–201.
26. Kent, A., Krebs, E. G. & Fischer, E. H. (1958) *J. Biol. Chem. 232*, 549–558.
27. Weilge, W. O. (1965) *J. Exp. Med. 121*, 289–307.
28. Weigle, W. O. (1971) *Clin. Exp. Immunol. 9*, 437–447.
29. Benyamin, Y. & Robin, Y. (1976) *Eur. J. Biochem. 62*, 15–19.
30. Haynes, A. L. & Lepow, I. H. (1964) *J. Immunol. 92*, 468–478.

S. J. Tzartos, Physiological Laboratory, University of Cambridge, Cambridge, Great Britain, CB1 2EG

A. E. Evangelopoulos*, Department of Biochemistry, National Hellenic Research Foundation, 48 Vassileos Constantinou Avenue, Athens 501/1, Greece

* To whom correspondence should be addressed.