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Purification and characterization of glycogen phosphorylase b from the breast muscle of fruit bat, *Eidolon helvum* Kerr

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ABSTRACT

The kinetic and physicochemical properties of glycogen phosphorylase b from the breast muscle of the fruit bat, *Eidolon helvum* Kerr were investigated in order to obtain some information about the possible physiological role of the enzyme in meeting the energy requirements of the bat muscle either at the initiation of or during flight. Glycogen phosphorylase b was purified from fruit bat breast muscle to apparent homogeneity with specific activity of approximately 37 units/mg of protein and a yield of 4 %. The enzyme was completely dependent on AMP for activity; hence it was designated phosphorylase b. The native and subunit molecular weights of the enzyme as determined by gel filtration on Sephadex G-200 and SDS-polyacrylamide gel electrophoresis were 187,000 ± 12,600 Da and 90,500 ± 1,200 Da respectively, thus implying that it is a dimeric protein. The Michealis-Menten constants, K_m, of the enzyme for glycogen and glucose-1-phosphate were 0.06 mg/ml and 6.94 mM respectively, while apparent K_m for AMP was 0.08 mM. The turnover number, k_{cat}, was 65.56 s^{-1}. Although sodium fluoride (NaF) and sodium sulphate (Na_2SO_4) on their own activated the enzyme, it was observed that in the presence of AMP, only sodium sulphate (Na_2SO_4) caused activation at low concentration. The optimum pH for the fruit bat muscle phosphorylase b activity was 6.4. In conclusion, the overall results of this study showed that the fruit bat muscle glycogen phosphorylase b is physically and catalytically similar to the enzyme from other mammalian sources.
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Keywords: fruit bat, glycogen, glycogen phosphorylase, breast muscle, animal flight

INTRODUCTION

Glycogen phosphorylase (α-1,4 glucan: orthophosphate glycosyl transferase, (EC 2.4.1.1) catalyzes the first step in the intracellular degradation of glycogen to release glucose molecules that are used for the provision of energy. Since its first isolation and crystallization by Green and Cori (1943), phosphorylase has attracted the interest of numerous researchers because it is the first enzyme in which activity was controlled by both covalent modification and allosteric interaction (Fischer et al., 1970). The enzyme has been found to exist in three distinct molecular forms or isozymes (Yonezawa and Hori, 1975, 1979; Hanabusa and Kohno, 1969, 1970) and it is found in many animal tissues, microorganisms and even in plants (Fischer and Krebs, 1958; San-Juan et al., 1993; Lin et al., 1995; Oluoha and Ugochukwu, 1995).

Fruit bats are involved in sustained non-stopped long distance migratory movement in the night despite their restless flying from one tree branch to the other during the day. Okon et al. (1978) have reported that carbohydrate formed the major diet of the frugivorous bats unlike the insect-eating ones. The movement of bats probably depends on glycogen as the main source of energy. It has

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been suggested that the glycogen deposited in breast muscle of the bat provides the energy requirement for the nocturnal flight in search of food (Okon et al., 1978; Okon and Ekanem, 1979). Holloszy et al. (1998) have also suggested that the rate of carbohydrate utilization during prolonged exercise is closely related to the energy needs of the working muscles.

In this report, we presented the isolation, purification and characterization of glycogen phosphorylase b from the breast muscle of the fruit bat, *Eidolon helvum*.

MATERIALS AND METHODS

Materials

DEAE-Sephacel, glucose-1-phosphate, adenosine-5-monophosphate (AMP), Fiske and SubbaRow reagent, β-glycerophosphate (disodium salt) and Molecular weight standards were purchased from Sigma Chemical Company, St. Loius, MO., USA. Blue dextran 2000 and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Sweden. All other reagents used were of analytical grade.

The fruit bats were collected from the Botanical Garden of Obafemi Awolowo University, Ile Ife, Nigeria. A fruit bat was rendered unconscious by a knock at the back of the head. The breast muscle was quickly excised and frozen until required.

Enzyme extraction and purification.

The purification was carried out following the procedure of Sevilla and Fischer (1969) with slight modification. The frozen muscle was weighed, minced with a pair of scissors, ground in a prechilled meat grinder and homogenized in Warring Blendor for one minute in 2.5 volumes of 1mM β-glycerophosphate buffer, pH 7.5 containing 1 mM EDTA and 2 mM β-mercaptoethanol. The homogenate was left standing at room temperature for 15 min with occasional stirring. This was later filtered through two layers of cheese cloth and then through a loose plug of glass wool into a beaker immersed in ice and allowed to stay for one hour with occasional gentle stirring.

The filtrate pH, which was 6.39, was lowered to pH 5.4 by the slow addition of 1 N acetic acid. The resulting turbid suspension was centrifuged at 4000 rpm for 40 min in an MSE Mistral 6000 refrigerated centrifuge. The supernatant obtained was filtered through coarse filter paper and the pH was raised to 6.8 by the addition of solid sodium hydrogen carbonate ($NaHCO_3$). The filtrate from the last step was brought to 50% ammonium sulphate saturation by the slow addition of solid ammonium sulphate (313 g/L) and left to stand for 18 hours. The resulting precipitate was collected by centrifugation at 4,000 rpm for 40 min. The precipitate obtained was dialyzed exhaustively at 4 ^{0}C against 5 mM β-glycerophosphate buffer, pH 7.0 containing 1 mM EDTA and 2 mM β-mercaptoethanol. The dialysate was centrifuged at 4,000 rpm to remove debris and other undissolved materials. The dialysate was concentrated by ultrafiltration using Amicon ultrafiltration unit with diaflo PM 30 membrane at 25 psi.

The concentrated dialysate was applied to a DEAE-Sephacel column (2.5 x 40cm). The column was first washed with 200 ml of 20 mM β-glycerophosphate buffer, pH 6.8 followed by elution with a linear salt gradient (0-0.5M NaCl) of 400 ml in 20 mM β-glycerophosphate buffer, pH 6.8. Fractions of 5ml were collected at a flow rate of 30 mlhr^{-1}. The active fractions were pooled and dialyzed against neutralized saturated ammonium sulphate in 40 mM Tris-HCl buffer, pH 7.4 containing 2 mM AMP and 20 mM magnesium acetate. The addition of AMP and magnesium acetate was to stabilize the enzyme in ammonium sulphate. The precipitate was collected by centrifugation at 4,000 rpm for 40 min.

The precipitate above was dialyzed in 5 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 7 mM β-mercaptoethanol and applied to a Sephadex G-200 column (2.5 x 100cm) that had been previously equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of 12 mlhr^{-1} into 4 ml fractions. The fractions containing phosphorylase b activity were pooled and concentrated by dialysis in 70% ammonium sulphate dissolved in 40 mM Tris-HCl buffer, pH 7.4 containing 2 mM AMP and 20 mM magnesium acetate.

Protein and enzyme assays

Protein concentration was determined by the method of Lowry et al. (1951) using

bovine serum albumin as standard. Glycogen phosphorylase b activity was assayed in the direction of glycogen synthesis (Sevilla and Fischer, 1969; Yonezawa and Hori, 1977, 1979) by measuring the rate of release of inorganic phosphate (P_i) from glucose-1-phosphate. A typical assay mixture contained, in final concentration, 20 mM β-glycerophosphate buffer pH 7.0, 1% glycogen, 50 mM glucose-1-phosphate, and 1 mM AMP and 0.02 ml of enzyme in a total volume of 0.5ml. Reaction was started by the addition of the enzyme. After incubation for 10 min at 30 °C, the reaction was stopped by the addition of 0.2 ml of 5% Trichloroacetic acid (TCA).

The inorganic phosphate released was analyzed by a modification of the Fiske and SubbaRow method (1925). This method involved the successive addition of 0.5 ml each of 5 N H_2SO_4 and 2.5% ammonium molybdate solution to the reaction mixture, which had been previously made up to 2.5 ml with distilled water. Subsequently, 0.15 ml of Fiske and SubbaRow reagent (1 g in 6.3 ml of distilled water) solution was added. The absorbance was read at 660 nm at 1 min intervals. Inorganic phosphate, (P_i), produced was intrapolated from a standard P_i curve. The standard P_i calibration curve was prepared by varying the concentration of Pi using 0.1 M K_2HPO_4 as standard. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1.0 μmole of P_i per minute at 30 °C.

Molecular weights determination

The molecular weight of the native enzyme was determined by gel filtration on a Sephadex G-200 column (2.5 x 100 cm). The standard proteins used were ferritin (480,000 Da), pyruvate kinase (237,000 Da), bovine globulin (160,000 Da), bovine serum albumin (66,000 Da), and ovalbumin (45,000 Da). The proteins were eluted with 5 mM Tris-HCl buffer, pH 7.5 at a flow rate of 12 $mlhr^{-1}$ and their elution volume, V_e, estimated. The void volume, V_o, was determined by the elution of Blue Dextran 2000 through the column. The molecular weight of glycogen phosphorylase b was interpolated from a plot of V_e/V_o against the molecular weight of the standards. The sub-unit molecular weight was determined by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) performed on 5% rod gel using the continuous phosphate buffer system of Weber and Osborn (1975). The molecular weight standards used were chymotrypsinogen (25,900 Da), aldolase (40,000 Da), catalase (57,000 Da), bovine serum albumin (66,000 Da) and β-glucosidase (90,000 Da).

Kinetics studies

The *initial* velocity study was performed by varying the concentrations of glycogen between 0.25 $mgml^{-1}$ and 10 $mgml^{-1}$ for a series of constant concentrations of glucose-1-phosphate between 4 mM and 50 mM according to the method of Florini and Vestling (1957). Similarly, the concentrations of glucose-1-phosphate was varied between 4 mM and 50 mM at different fixed concentrations of glycogen (0.25 $mgml^{-1}$ - 5.0 $mgml^{-1}$). The kinetic constants were evaluated from the secondary plots.

Effect of adenosine monophosphate (AMP)

Effect of AMP on the activity of the enzyme was studied by assaying the enzyme at various concentrations of AMP ranging from 0.02 mM to 1.0 mM while glycogen and glucose-1-phosphate concentrations were kept at 10 $mgml^{-1}$ and 50 mM respectively.

Effect of pH

The buffers used were 2-(N-Morpholino) ethane sulphonic acid [MES] (pH 5.0 - 6.4), Tris-maleate (pH 6.4 - 6.8) and N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid [HEPES] (pH 6.6 - 7.8). The enzyme was assayed in a reaction mixture, which contained 66.7 mM buffer, 66.7 mM glucose-1-phosphate, 0.67 % glycogen, 5 mM AMP and 0.02 ml of enzyme solution.

Effect of salts

Effects of sodium fluoride (NaF) and sodium sulphate (Na_2SO_4) were investigated by assaying 0.02 ml of the enzyme for 10 min at 30 °C in 20 mM β-glycerophosphate buffer, pH 7.0 containing 50 mM glucose-1-phosphate, 1% glycogen with or without 5 mM AMP and various concentrations of NaF and Na_2SO_4.

RESULTS

Enzyme purification

The summary of a typical purification procedure for glycogen phosphorylase b from the breast muscle of fruit bat is presented in Table 1. The DEAE-Sephacel chromatography step produced a peak of phosphorylase b activity (Figure 1). The fraction when further purified by gel filtration on Sephadex G-200 yielded a homogenous protein (adjudged by the presence of a single band of protein after electrophoresis in the presence of SDS (data not shown)) with a specific activity of 37 units/mg of protein. This represents a purification fold of 100.

The purified enzyme was designated phosphorylase b because it was found to be completely dependent on AMP for activity. There was no trace of phosphorylase a in the preparation because no enzymatic activity was observed when the preparation was assayed in the absence of AMP. The inclusion of EDTA, which inhibits phosphorylase kinase that converts phosphorylase b to phosphorylase a, in all buffer reduces the possibility of the presence of phosphorylase a in the final enzyme preparation. The addition of AMP and magnesium acetate stabilizes the enzyme when stored as ammonium sulphate precipitate. A rapid loss of enzymatic activity was observed when stored without AMP and magnesium acetate in ammonium sulphate.

Molecular weight determination

The molecular weight of the native enzyme estimated by gel filtration on Sephadex G-200 was 187,000 ± 12,600 Da. The subunit molecular weight was 90,500 ± 1,200 Da. Thus, the fruit bat glycogen phosphorylase is a dimer.

Kinetic studies

A typical Lineweaver-Burk plot of the initial reaction velocity against the concentrations of glycogen at fixed concentrations of glucose-1-phosphate is as shown in Figure 2A. The secondary slope and intercept plots are shown in the Figures 2Bi and ii according to Florini and Vestling (1957) and Cleland (1970). The kinetic constants obtained for the enzyme from the secondary plots are summarized in Table 2. Figure 3 shows the Lineweaver-Burk plot of the effect of AMP concentration on the activity of the enzyme. The K_m value for AMP was 0.08 mM.

Effects of salts

Figure 4 shows the results of the effect of salts on the activity of fruit bat breast muscle phosphorylase. NaF concentration of 0.1 M produced more than 100% activation in the absence of AMP. Above this concentration there was a great reduction in the activity of the enzyme. In the presence of AMP, NaF was found to inhibit the enzyme activity. Up to 70% inhibition was recorded with 0.3 M NaF. The fruit bat muscle phosphorylase b was slightly activated by low concentration of Na_2SO_4 in the presence of AMP but highly activated in the absence of AMP. About 3 fold increase in the enzyme activity was observed with 0.3 M Na_2SO_4.

Table 1: Summary of the purification procedure of glycogen phosphorylase b from fruit bat breast muscle.

Fractions	Volume (ml)	Total activity (units/min)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	965	22,616.8	66,520	0.34	100	1
Neutralized acid supernatant	710	14,833.5	10,230	1.45	65.5	4.3
50% ammonium sulphate precipitate	18	8,125	650	12.50	35.9	36.8
DEAE-Sephacel ion exchange chromatography	97	4,111	232	17.72	18.2	52.1
Gel filtration Sephadex G-200	63	930	25	37.2	4.1	109.4

Table 2: Kinetic parameters of glycogen phosphorylase b from fruit bat breast muscle.

Parameters	Values
Michaelis-Menten constant for glycogen (K_m^{GLY})	0.06 $mgml^{-1}$
Michaelis-Menten constant for glucose-1-phosphate (K_m^{G1P})	6.94 mM
Maximum Velocity (V_{max})	34.5 $\mu molemin^{-1}ml^{-1}$
Dissociation constant for glycogen (K_i^{GLY})	1.13 $mgml^{-1}$
Dissociation constant for glucose-1-phosphate (K_i^{G1P})	30.2 mM
Turnover Number $\{K_{cat}\}$	65.56 s^{-1}
Specificity constant $\{K_{cat} / K_m^{GLY}\}$	1.1 X 10^3 $M^{-1}s^{-1}$
Specificity constant $\{K_{cat} / K_m^{G1P}\}$	9.45 X 10^3 $M^{-1}s^{-1}$

The kinetic parameters were determined from the slope and intercept replots of the primary double reciprocal plots (Fig. 3) according to Florini and Vestling (1957) and Cleland (1970) for a bireactant reaction following a sequential mechanism. The initial velocity study was performed by varying the concentrations of glycogen from 0.25-10.0 $mgml^{-1}$ at fixed concentrations of glucose-1-phosphate (4–50 mM). Similarly, the concentrations of glucose-1-phosphate were varied between 4 mM and 50 mM at fixed concentrations of glycogen (0.25-5.0 $mgml^{-1}$). Results were the average of two separate determinations.

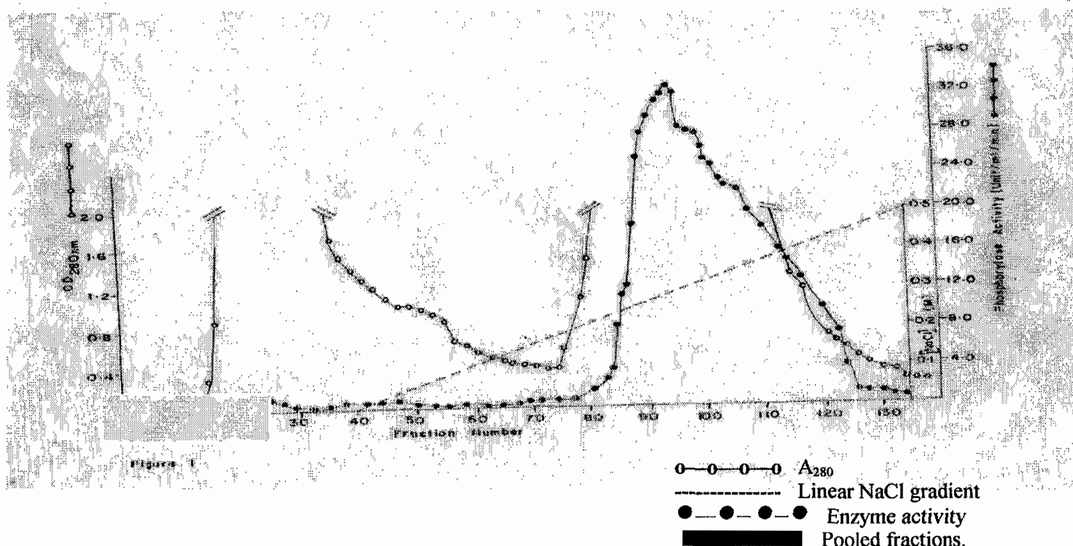

Figure 1: The elution profile of fruit bat breast muscle glycogen phosphorylase on DEAE-Sephacel. The concentrated dialysate of 50% ammonium sulphate of the acid neutralized supernatant was applied to the column (2.5 X 40 cm). The enzyme was eluted with a linear salt gradient of 0.0-0.5 M NaCl in 20 mM glycerophosphate buffer, pH 6.8, at a flow rate of 30 $mlhr^{-1}$ and fractions of 5 ml was collected.

DISCUSSION

The purification of glycogen phosphorylase b from the breast muscle of the fruit bat *Eidolon helvum,* Kerr, is presented. The procedures involved ammonium sulphate precipitation, ion exchange chromatography and gel filtration on Sephadex G-200. The purified enzyme was found to be completely dependent on AMP for activity, hence, it was designated phosphorylase b. Storage of the enzyme in ammonium sulphate without AMP and/or magnesium acetate results in the loss of enzyme activity. Consequently, the purified enzyme was stored as ammonium sulphate suspension containing AMP and magnesium acetate. Cori et al. (1943) had earlier observed that ammonium sulphate exhibited an inhibitory effect on phosphorylase but this inhibition can be counteracted by the addition of small amount of AMP and/or magnesium acetate. Thus, the stability of the enzyme in ammonium sulphate can be greatly improved with the addition of AMP. Johnson et al. (1979) had discussed that the structural basis for the stabilizing effect of AMP on phosphorylase b is due to the interaction of some residues with AMP that holds the monomers together. The specific activity of fruit bat glycogen phosphorylase b (37 units

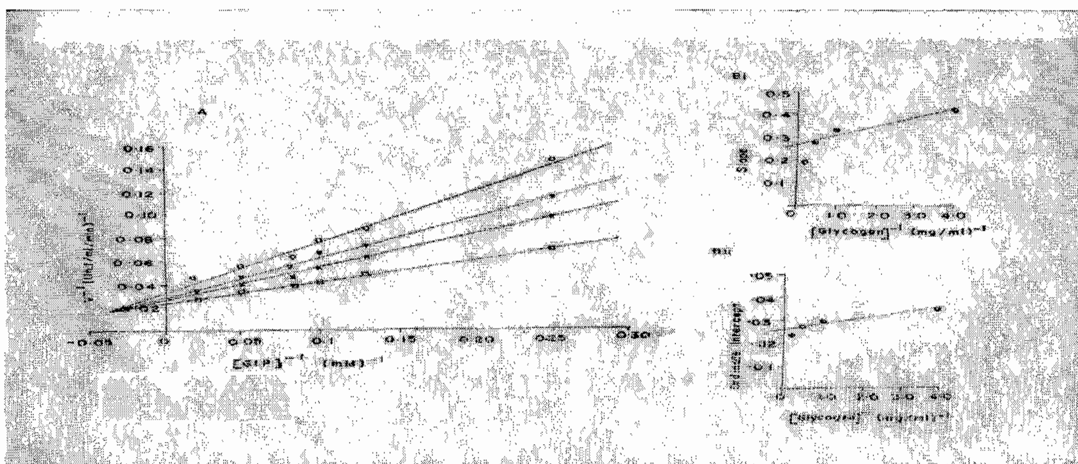

Figure 2A: Effect of glycogen concentration on the activity of fruit bat breast muscle glycogen phosphorylase. The Lineweaver-Burk plots showing the effect of varying concentration of glycogen at different fixed concentrations of glucose-1-phosphate (G1P). The reaction mixture of 0.5 ml contained 20 mM glycerophosphate buffer, pH 7.0, 5 mM AMP, glycogen concentration between 0.25 $mgml^{-1}$ and 10.0 $mgml^{-1}$, the indicated amount of glucose-1-phosphate and 20 µl of enzyme solution (1.64 µg), 4 mM (□—□—□), 6 mM (△—△—△), 10 mM (•—•—•), 20 mM (x—x—x—x) and 50 mM (o—o—o—o).

(B) Secondary plots of data from Lineweaver-Burk plot showing the effect of varying glucose-1-phosphate concentration on the enzyme activity.

(i) Replot of slope versus 1/[GIP]

(ii) Replot of ordinate intercept versus 1/[GIP]

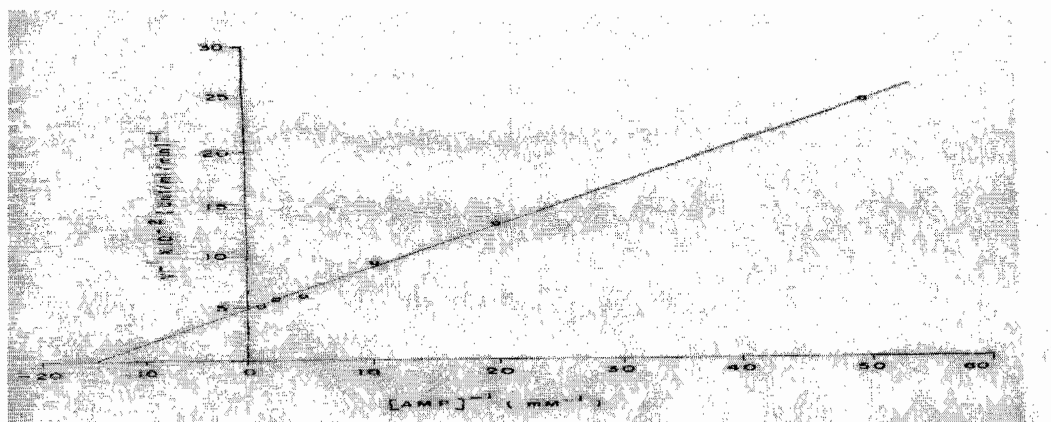

Figure 3: Effect of concentration of AMP on glycogen phosphorylase b from the breast muscle of fruit bat. Assays were carried out in 20 mM glycerophosphate buffer, pH 7.0, 10 $mgml^{-1}$ glycogen, 50 mM glucose-1-phosphate and various concentration of AMP ranging from 0.0 - 1.0 mM and a constant amount of enzyme.

per mg of protein) is lower than that from rat skeletal muscle (Sevilla and Fischer, 1969) and skate muscle (Yenozawa and Hori, 1979) but of the same magnitude with the value obtained for the same enzyme from abdominal muscle of crayfish (Hergenhahn, 1983).

The native and subunit molecular weights of the purified enzyme are 187,000 ± 12,600 Da and 90,500 ± 1,200 Da respectively, thus, suggesting that the glycogen phosphorylase b of fruit bat is a homodimer. The enzymes from frog skeletal muscle (Metzger et al., 1968), skate and lamprey muscle (Yonezawa and Hori, 1977; 1979), rat skeletal muscle (Sevilla and Fischer, 1969) and bovine spleen (Kamogawa and Fukui, 1971) and from several other sources (Childress and Sacktor, 1970; Cohen et al., 1971; Yacoub et al., 1983) have been found to be dimers with similar molecular weights.

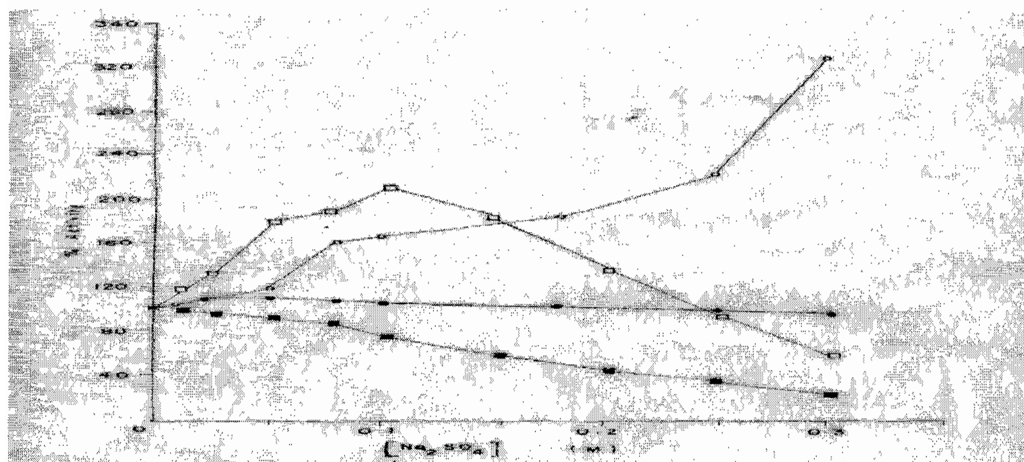

Figure 4: Effect of sodium fluoride (NaF) and sodium sulphate (Na_2SO_4) on the activity of glycogen phosphorylase of fruit bat breast muscle. The enzyme was assayed with various concentrations of NaF and Na_2SO_4 with or without 5 mM AMP in 0.5 ml reaction mixture containing 20 mM glycerophosphate buffer (pH 7.0), 50 mM glucose-1-phosphate and 1% glycogen. The effect of NaF without AMP (O) and with 5 mM AMP (●). The effect of Na_2SO_4 without AMP (□) and with 5 mM AMP (■).

The linear and intersecting patterns of the double reciprocal plots typified by Fig. 3 are consistent with sequential reaction mechanism in which both substrates must bind to the enzyme at the same time to form a ternary complex before product formation can occur. The secondary plots of these data (insets of Figs 3 and 4) were linear and yielded kinetic constants which are summarized in Table 2 (Florini and Vestling, 1957; Cleland, 1970). It has been shown by Johnson et al. (1992) that it is only when the ternary-complex is formed between enzyme, glucose-1-phosphate and glycogen that the correct juxtaposition of phosphates is promoted for catalysis to occur. In other words, both substrates must bind to the enzyme simultaneously before product can be formed. Earlier kinetic results on rabbit liver phosphorylase (Maddaiah and Madsen, 1966) and rabbit muscle phosphorylase (Engers et al., 1969) have shown that phosphorylase exhibited rapid equilibrium random Bi-Bi mechanism.

Some salts (NaF, Na_2SO_4, KCl, NaCl, $KClO_3$) have been reported to affect the enzyme activity *in vitro* (Cowgill, 1959; Stalmans and Hers, 1975; Yonezawa and Hori, 1976, 1977; Miller et al., 1975). While fruit bat muscle glycogen phosphorylase b was activated by NaF in the absence and presence of AMP, the enhancement of activity was more remarkable in the absence of the nucleotide. This activation by NaF in the absence of AMP has been reported for the enzyme from several vertebrates (Sealock and Graves, 1967, Stalmans and Hers, 1975; Lederer and Stalmans, 1976; Peterson, 1981). However, crayfish muscle phosphorylase b has been reported to lose activity in the presence of both AMP and NaF (Hergenhahn, 1983). The activation of the fruit bat muscle enzyme by sodium sulphate in the absence of AMP, suggests that the inorganic salt could be a substitute for the nucleotide. The molecular mechanism of stimulation by Na_2SO_4, which has been observed with the enzyme from other sources (Engers and Madsen, 1968; Stalmans and Hers, 1975; Petersen, 1981), is not fully understood.

The Michaelis-Menten constant value for glycogen (0.06 $mgml^{-1}$) obtained for the fruit bat enzyme is similar to that of insect flight muscle phosphorylase (Childress and Sacktor, 1970). This is interesting considering the fact that each source of the enzymes is flight muscle, which plays a significant role in the initiation and sustenance of flight. In support of the involvement of glycogen phosphorylase b in the provision of energy required at the initiation of flight, a decrease in ATP concentration, and an increase in ADP, inorganic phosphate and AMP concentrations have been observed at the

onset of muscle contraction in insect (Sacktor and Hurlbut, 1966). This situation would favour a rapid increase in the rate of respiration and other parameter like phosphorolysis of glycogen during the transition from a muscle at rest to one carrying out intense work like that of fruit bat flight muscle (Sacktor and Hurlbut, 1966). Okon et al. (1978) have reported an increase in the glycogen level in the breast muscle of fruit bat prior to the nocturnal flight. Newsholme and Leech (1983) have also observed that if the carbohydrate stored in muscle can be elevated prior to the muscle contraction, a given level of exercise or muscle contraction can be maintained for longer period of time. It is, thus, likely that the role of glycogen phosphorylase is to provide the energy needed for such muscle contraction.

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