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A NOVEL INHIBITOR OF GLYCOGEN PHOSPHORYLASE FROM CRAYFISH HEPATOPANCREAS

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Abstract—1. A novel glycogen phosphorylase inhibitor was partially purified from crayfish hepatopancreas.

2. The inhibitor was found only in two species of crayfish examined, and not in lobster, fresh and salt water clams, mussels or cockroaches.

3. The inhibitor is a small protein ($M_r = 23{,}000$) which did not show proteolytic activity.

4. Preliminary kinetic analysis of the inhibitory mechanism indicated that it bound to both glycogen and the glycogen phosphorylase protein.

5. Inhibitor binding to glycogen resulted in a competitive inhibition pattern with respect to glycogen phosphorylase (inhibition constant of *ca* 10 μg/ml).

6. The inhibitor also bound glycogen phosphorylase directly with a binding coefficient of 100 μg/ml resulting in a partially non-competitive inhibition pattern with respect to phosphate.

INTRODUCTION

Glycogen phosphorylase is a key enzyme involved in the regulation of glycogen metabolism and blood glucose levels. Consequently, several complex mechanisms have evolved to control its activity including: reversible phosphorylation of its enzyme subunits (which changes the inactive *b* form into the active *a* form, see Cohn, 1980; Hers, 1976; Storey, 1985), allosteric modification of enzyme activity (Aragon *et al.*, 1980; Dombradi, 1981; Engers *et al.*, 1969, 1970a,b; Hers, 1976; Vaandrager *et al.*, 1987) and control of total available enzyme activity (Storey and Storey, 1984, 1985). Overall control of glycogen phosphorylase activity is maintained by a balance between these three general mechanisms which combine to increase activity [e.g. during starvation in liver, see Hers (1976) and freezing in wood frogs, Storey and Storey (1984)] or decrease activity [e.g. during anoxia in goldfish liver, Storey (1987)] depending on metabolic demand.

During our studies of glycogen phosphorylase regulation in the crayfish *Orconectes virilis*, we discovered that (a) hepatopancreas apparently had no glycogen phosphorylase activity, and (b) extracts of hepatopancreas inhibited glyocgen phosphorylases from other sources. These results led us to examine the possibility that a specific glycogen phosphorylase inhibitor existed in crayfish hepatopancreas.

Previous studies of glycogen phosphorylase activity in the hepatopancreas of crustaceans and molluscs was hampered by the presence of proteases, which interfered with total enzyme activity measurements (Lackner, 1985; Rosell *et al.*, 1987; Wang and Scheer, 1963). Proteolytic degradation is rapid in some cases, with only half of the measured enzyme activity remaining after 40 min in some preparations (Rosell *et al.*, 1987). These proteases are not inhibited by the addition of general protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) but are apparently species specific and require specific inhibitors. For example, soybean and lima bean trypsin inhibitors, as well as leupeptin, are required to protect glycogen phosphorylase against lobster hepatopancreas proteases (Rosell *et al.*, 1987) and leupeptin and antipain are required to protect pyruvate kinase from degradation by snail hepatopancreas (Wang and Scheer, 1963). In contrast to these results, the present study indicates that the *O. virilis* glycogen phosphorylase inhibitor (GPI) from hepatopancreas is not a protease but a specific inhibitor of glycogen phosphorylase activity. This paper presents the partial purification and characterization of this novel glycogen phosphorylase inhibitor.

MATERIALS AND METHODS

(a) Chemicals and enzymes

All chemicals and enzymes were purchased from Sigma Chemical Co. (St Louis, Mo.) or from Boehringer Mannheim (Montreal, P.Q.) unless otherwise indicated. Rabbit skeletal muscle glycogen phosphorylase *a* (type P-1261) and protease (subtilisin, type XXIV) were obtained from Sigma and used without further purification. α-Amylase, β-Amylase, glucoamylase and cellulase were obtained from Finnsugar Biochemicals Inc. (Schaumburg, Ill.), dextranase was obtained from Novo Industri A/S (Bagsvaerd, Denmark) and DNAse I, RNAse, Eco R1 and Hind III were graciously supplied by Dr D. Hickey (Ottawa University).

(b) Animals

The two species of crayfish used in this study, *O. virilis* and *Cambarus bartoni*, were obtained from local streams

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Abbreviations: GPI, glycogen phosphorylase inhibitor; IU, international unit of activity (1 IU = 1 μmol/min); PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

and kept in dechlorinated water at 10°C for at least two weeks prior to killing. Animals were fed small pieces of trout muscle and gut daily. Fresh water clams (*Anodonta cygnea*) were obtained from the Ottawa river and used immediately. Lobsters (*Homarus americanus*), salt water clams (*Mercenaria mercenaria*), and mussels (*Mytilus edulis*) were obtained from a local fish market and used immediately. Cockroaches (*Periplaneta americana*) were obtained from the Carleton University colony.

(c) Measurement of GPI inhibitory activity

Unless otherwise indicated, GPI activity was measured by adding crude GPI (see section g) to glycogen phosphorylase in 50 mM potassium phosphate buffer containing: 15 mM $MgCl_2$, 0.25 mM EDTA, 10 μM glucose 1,6-bisphosphate, 0.4 mM NADP, 1.6 mM AMP (Basic Assay Buffer) with 0.7 IU phosphoglucomutase and 0.2 IU glucose 6-phosphate dehydrogenase. The reaction was initiated by adding 2 mg/ml glycogen, and measured, after a steady-state had been achieved, by following NADPH appearance at 340 nm, at 22°C in a 1 ml cuvette.

(d) Determination of the kinetic constants for glycogen phosphorylase in the presence of GPI

The kinetic constants for glycogen phosphorylase were determined in Basic Assay Buffer containing 1.6 mM AMP, 0.7 IU phosphoglucomutase and 0.2 IU glucose 6-phosphate dehydrogenase. The K_m value for phosphate was measured with 50 mM imidazole (pH 7.0) substituted for potassium phosphate and 2 mg/ml glycogen. The reaction was initiated by the addition of glycogen and the initial rates were measured at 22°C by following NADPH appearance at 340 nm.

(e) Other enzyme assays

Hexokinase, phosphofructokinase, aldolase, pyruvate kinase and lactate dehydrogenase were assayed as described previously (Brooks and Storey, 1987). Phosphoglucose isomerase (from *O. virilis* tail), phosphoglucomutase and glucose 6-phosphate dehydrogenase were assayed in Basic Assay Buffer containing either 10 mM fructose 6-phosphate, for phosphoglucose isomerase, or 0.5 mM glucose 1-phosphate, for phosphoglucomutase and glucose 6-phosphate dehydrogenase combined. All assays were performed at 340 nm and 22°C.

(f) Other procedures

Isoelectric focusing of GPI samples was performed at 4°C according to Vesterberg (1971). A gradient of pH 3.5 to pH 10 of LKB 1089 Ampholine was stabilized by a 0–30% linear sucrose gradient. Native gel electrophoresis was performed on a 12% polyacrylamide gel (non-SDS) according to Laemlli (1970). Gels were stained with silver nitrate (Nielson and Brown, 1984) and scanned on a densitometer. Protein determinations were performed using the Bio-Rad Protein Assay kit (Bio-Rad, Mississauga, Ont.) with bovine serum albumin as a standard (Bradford, 1976).

GPI was derivatized by reaction with Methylene Blue by a procedure modified by Tsai *et al.* (1985). Briefly, 200 μl of 10 mg/ml crude homogenate was mixed with 10 μl Methylene Blue to give a final concentration of either 0.5 or 10 mM. This mixture was incubated overnight at room temperature in a glass test tube positioned approximately 6 cm from a 25 W light bulb. Experiments to test the disappearance of GPI activity included a control consisting of Methylene Blue at the same concentrations. The results of these control experiments showed that methylene blue alone had no effect on glycogen phosphorylase activity.

Gel exclusion chromatography using Sephadex G-100 for the determination of the GPI M_r value was performed in Column Buffer (50 mM imidazole, pH 7.0, 25 mM β-mercaptoethanol, 100 mM KCl and 30% (v/v) glycerol) at 22°C. A 0.3 ml aliquot of the crude GPI (in Column Buffer) was loaded on a 15 ml Sephadex G-100 column (0.4 cm dia × 30 cm) and 0.3 ml fractions were collected. The M_r of the protein was determined by plotting the reciprocal error function of the partition coefficient as a function of the logarithm of the M_r of various standards according to Suelter (1985). Molecular weights of the standards were: Aldolase, 158,000; bovine serum albumin, 67,000; ovalbumin, 44,000; chymotrypsinogen A, 25,000; ribonuclease A, 13,700; and cytochrome *c*, 13,500.

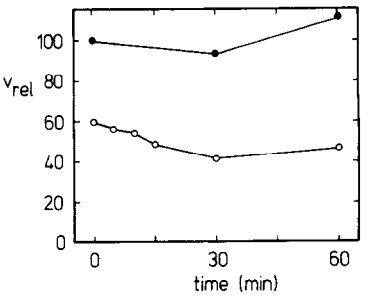

Fig. 1. Time course for GPI action. Either rabbit muscle glycogen phosphorylase *a* (0.25 mg/ml, ●) or rabbit muscle glycogen phosphorylase *a* and crude GPI (9.6 mg/ml, ○) were incubated at room temperature in 200 mM glucose, 40 mM imidazole (pH 7.0), and 20 mM β-mercaptoethanol. At the indicated time periods, 5 μl of the reaction mixture was removed and assayed for glycogen phosphorylase activity.

(g) Purification procedure

Approximately 1.6 g of *O. virilis* hepatopancreas tissue (from four individuals) was rapidly removed from decapitated crayfish and homogenized (1:1) in ice cold 250 mM sucrose, 50 mM imidazole (pH 7.0), 25 mM β-mercaptoethanol, 0.5 mM EDTA and 50 μM PMSF using 20 strokes of a loosely fitting Potter–Elvehjem homogenizer. This homogenate was centrifuged at 15,000 ***g*** for 15 min and the supernatant was filtered through cheese cloth (crude homogenate) and placed in the middle portion of a 100 ml isoelectric focusing column. The column was allowed to develop overnight at 300 V (constant voltage) and the samples collected and assayed for activity. The peak inhibitory activity occurred at a pH value of *ca* 4.1. The three fractions with the highest inhibitory activity were collected, concentrated six-fold by dialysis against solid sucrose, and brought up to 30% glycerol, 0.1 M NaCl, 50 mM imidazole (pH 7.0) and 25 mM β-mercaptoethanol. An aliquot (0.15 ml) of this sample was loaded on a 10 ml Sephadex G-100 column preequilibrated with the same buffer and 148 μl fractions were collected. The GPI activity was typically greatest in the 29th fraction. This sample was then subjected to electrophoretic analysis.

RESULTS

(a) Preliminary characterization

Initial measurements of glycogen phosphorylase activity in *O. virilis* hepatopancreas and gill homogenates showed that little or no activity was associated with these tissues (data not shown). This unexpected result was further investigated by adding hepatopancreas or gill crude homogenate to tail homogenates which had a high glycogen phosphorylase activity. Interestingly, hepatopancreas and gill homogenates inhibited the tail glycogen phosphorylase activity, suggesting that a glycogen phosphorylase inhibitor was present in these tissues.

Preliminary investigation into the mode of action of the GPI from hepatopancreas indicated that it did not act by proteolytically degrading glycogen phosphorylase. This was shown by measuring glycogen phosphorylase activity after increasing durations of GPI plus glycogen phosphorylase *a* incubation. The data of Fig. 1 show that glycogen phosphorylase *a* activity does not decrease with longer periods of glycogen phosphorylase *a* plus GPI incubation. That GPI does not act by proteolytic degradation is also shown by data demonstrating the reversibility of the inibiting; sequential dilution of a GPI–glycogen phosphorylase mixture (which had been pre-incubated for 1 hr) resulted in a proportional decrease in the degree of inhibition. Thus, diluting the concentration of an enzyme plus GPI mixture reduced (and reversed) the GPI inhibitory effect. The data of Fig. 1 also demonstrate the stability of GPI to possible (secondary) proteolytic degradation by endogenous hepatopancreas proteases.

The data of Table 1 show that GPI from *O. virilis* inhibited glycogenphosphorylase (from muscle tissue) from a variety of different animals, including rabbit, lobster, clam, mussel and cockroach. In all cases, GPI equally inhibited both glycogen phosphorylation *a* and *b* (data not shown). GPI did not, however, inhibit the activity of several other glycolytic enzymes; the activity of glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, phosphoglucomutase, hexokinase, aldolase, phosphofructokinase, pyruvate kinase and lactate dehydrogenase were not affected by GPI. Interestingly, inhibitory activity was only associated with the two species of crayfish tested (*O. virilis* and *C. bartoni*), and was not present in the hepatopancreas of lobster; mussel; fresh water or salt water clam; or cockroach.

The results of several experiments to determine the physical nature of the inhibitor are shown in Table 2. Of all the treatments listed, only three reduced the GPI inhibitory activity: boiling for 10 min, decreasing the pH to 1 followed by centrifugation, and treatment with Methylene Blue. These three methods are commonly used to destroy protein activity: high acid causes protein precipitation, boiling denatures proteins and Methylene Blue derivatizes amino acid residues (principally histidyl residues). These results, combined with the lack of any effect of the enzymes listed in Table 2, suggest that the GPI is a protein rather than a sugar or nucleic acid polymer. Furthermore, the lack of any inhibitory effect of added protease (and the apparent lack of hepatopancreas proteolytic activity toward GPI in crude form) suggest that GPI is a relatively small molecular weight protein. Experiments with Ca^{2+} and EGTA showed that calcium ions are not required for inhibitory activity. The results of experiments performed in the presence of glycerol and triton X-100 showed that the inhibitory activity of GPI does not depend on hydrophobic interactions.

Table 1. Effect of hepatopancreas GPI on glycogen phosphorylases from several animals

Source	Crude homogenate required to inhibit 50% of control activity (mg)
Crayfish tail (*O. virilis*)	0.128
Rabbit muscle *a*	0.287
Lobster tail	0.179
Lobster heart	0.168
Mussel foot	0.146
Clam foot (salt water)	0.079
Cockroach flight muscle	0.092

Control activity of glycogen phosphorylase from all sources was adjusted to *ca* 3.4 mIU/ml mix. Glycogen phosphorylase from rabbit muscle (type *a*) was obtained from Sigma Chemical Co. All other enzymes were measured in crude homogenates of the tissues indicated.

Table 2. Effect of various treatments on the inhibitory activity of crude GPI from *O. virilis* hepatopancreas

Treatment	Remaining glycogen phosphorylase activity after addition of GPI (%)
None	5
Boil 10 min	75
Desalt[a]	1
pH to 1, centrifuge	60
pH to 13, centrifuge	2
Ethanol extract	1
Methanol/$CHCl_3$ extract	1
148 IU trypsin	8
172 IU chymotrypsin	2
108 IU papain	5
518 IU pepsin	5
108 IU protease	6
100 IU α-amylase	1
100 IU β-amylase	1
100 IU amyloglucosidase	4
15 IU β-glucosidase	6
100 IU cellulase	4
110 IU dextranase	1
205 IU isomaltase	5
105 IU isoamylase	2
1 mg RNAse	6
1 IU DNAse I	12
1 IU Eco RI	6
1 IU Hind III	7
0.5 mM Methylene Blue	35
10 mM Methylene Blue	62
10 mM $CaCl_2$	10
10 mM EGTA	10
30% Glycerol	8
1% Triton X-100	6

Control activity was measured using 8 mIU of crude *O. virilis* tail glycogen phosphorylase. Percent remaining activity represents the activity remaining after addition of the equivalent of 0.25 mg of crude GPI homogenate to each assay tube. Enzyme digests were carried out by adding the indicated number of units of enzymes to 200 μl of crude GPI (prepared by homogenizing hepatopancreas in 250 mM sucrose, 20 mM imidazole (7.0), 10 mM β-mercaptoethanol at a 1:4 (w:v) dilution), and incubating for 16 hr at 30°C. The GPI activity of this preparation was measured (after the addition of 50 μM PMSF to the protease tubes) by adding 20 μl aliquots of the treated sample to a crude *O. virilis* tail glycogen phosphorylase preparation. Experiments with $CaCl_2$, EGTA, glycerol and Triton X-100 were performed by adding GPI to glycogen phosphorylase in buffer plus effector.

[a]The crude protein was desalted using a spun column technique (Helmerhorst and Stokes, 1980). Values represent the mean of two separate determinations.

(b) Kinetic characterization

The results presented above demonstrated the proteinatious nature of GPI, as well as the reversibility of its action, but they did not indicate the mechanism of inhibition. In order to determine the kinetic pattern of GPI inhibition, rabbit muscle

glycogen phosphorylase *a* activity was measured in the presence of increasing GPI at varying concentrations of glycogen or phosphate. The data of Figs 2A and B show initial enzyme activities determined at varying substrate concentrations and at 1.6 mM AMP; AMP is required to obtain linear Lineweaver–Burk plots (Engers *et al.*, 1970a,b). Visual inspection of these figures shows that GPI was competitive with respect to glycogen at saturating phosphate (all lines intersect at a common V_{max} value) and partially non-competitive with respect to phosphate at saturating glycogen (all lines intersect in the second quadrant). The inhibition was linear in both cases, and the slopes of the lines in Fig. 2 gave the inhibition constants. Thus, as Table 3 indicates, the K_I value (competitive inhibition constant from Fig. 2A) obtained by varying glycogen concentrations is 4 μg/l, the K_{II} value (non-competitive inhibition constant determined from Fig. 2B) obtained by varying phosphate is 99 μg/ml, and the K_{IS} value (non-competitive inhibition constant from Fig. 2B) is 13 μg/ml. These results are not consistent with any simple pattern of inhibition (Cleland, 1963a,b; Plowman, 1972) and suggest that the inhibition may be complex, or involve multiple sites of GPI action.

The experiment of Fig. 3 was designed to further resolve the mode of GPI action. Since the competitive inhibition observed in Fig. 2 could result from GPI binding either to glycogen or to glycogen phosphorylase, GPI was pre-incubated either by itself (tracing b, Fig. 3), with glycogen phosphorylase *a* (tracing c, Fig. 3) or with glycogen (tracing d, Fig. 3). Note that when GPI was incubated with glycogen, the inhibition was almost immediate, whereas a lag period of 5–6 min was required before inhibition occurs in tracing b or c (Fig. 3). This suggests that GPI primarily binds glycogen (and not glycogen phosphorylase) so that it competes with glycogen phosphorylase *a* for glycogen.

(c) Partial purification and characterization

A partial purification of *O. virilis* GPI was achieved in a three step procedure and the results are presented in Table 4. The crude homogenate represents the

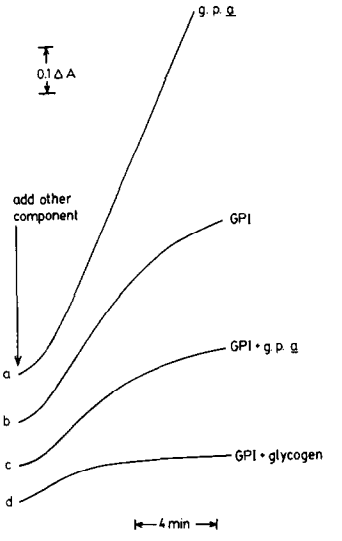

Fig. 3. Time course for inhibition: effect of different incubation regimens. Either (a) glycogen phosphorylase *a*, (b) 0.16 mg crude GPI, (c) 0.16 mg crude GPI plus 5 μg glycogen phosphorylase *a*, or (d) 0.16 mg crude GPI plus 2 mg glycogen were incubated for 15 min in Basic Assay Buffer. The reaction was initiated by the addition of (a) glycogen, (b) glycogen phosphorylase *a* and glycogen, (c) glycogen or (d) glycogen phosphorylase *a*.

Table 3. Kinetic constants for crude GPI inhibition of glycogen phosphorylase *a* from rabbit skeletal muscle

Kinetic constant	Varied substrate	Constant substrate	Plus GPI?	Value
K_m	Glycogen	Phosphate	No	0.04 mg/ml
K_m	Phosphate	Glycogen	No	0.2 mM
K_I	Glycogen	Phosphate	Yes	4 μg/ml
K_{IS}	Phosphate	Glycogen	Yes	13 μg/ml
K_{II}	Phosphate	Glycogen	Yes	99 μg/ml

Values are determined from Fig. 2. The K_I value represents the inhibition constant for GPI determined from the slopes of Fig. 2A assuming competitive inhibition. The K_{II} value represents the inhibition constant for GPI determined from the intercepts of Fig. 2B, and the K_{IS} value represents the inhibition constant for GPI determined from the slopes of Fig. 2B (assuming partially non-competitive inhibition). Nomenclature is from Plowman (1972). Values represent the mean of two determinations.

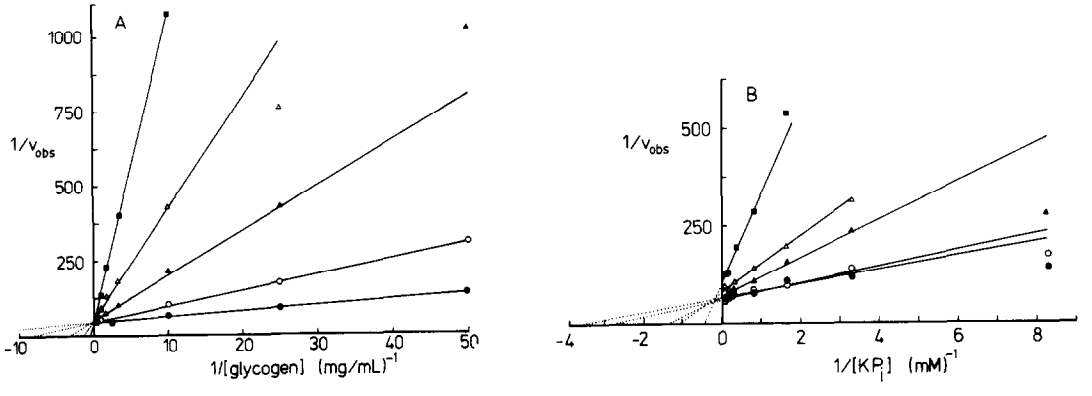

Fig. 2. Effect of crude GPI on glycogen phosphorylase *a* kinetic parameters: Lineweaver–Burk plots of velocity versus substrate concentration. The assay contained 5 μg rabbit muscle glycogen phosphorylase *a* in Basic Assay Buffer and varying amounts of GPI, glycogen (graph A) and phosphate (graph B). ●, control; ○, 0.013 μg crude GPI; ▲, 0.037 μg crude GPI; △, 0.061 μg crude GPI; and ■, 0.123 μg crude GPI. Assay conditions as described in Materials and Methods.

supernatant obtained after centrifugation, and filtration through cheese cloth. It is desirable to homogenize the hepatopancreas at a 1:1 dilution with sucrose buffer since this allows one to load a minimal volume on the IEF column. The isoelectric focusing step gives a 18-fold purification of the crude homogenate with no apparent loss of activity. The three fractions with the highest inhibitory activity were collected and concentrated for the next step, and so the apparent loss in the isoelectric focusing step comes from discarding fractions without high activity. Several proteins precipitate at a pH value of 5, which is close to the pI value of 4.07 obtained from Fig. 6, so one must be careful when pooling the fractions from this column. The Sephadex G-100 column step results in a further 13-fold purification, but did not give a purified protein as shown by native gel electrophoresis (data not shown). It is important to note that the G-100 column was equilibrated with buffer containing 0.1 M NaCl and 30% glycerol. These substances were added to prevent loss of GPI, which binds to the column matrix in their absence. Thus, in the absence of salt and glycerol, >95% of the GPI remains on the column, and cannot be recovered.

It is possible to obtain an estimate of the molecular weight of GPI by calibrating a Sephadex G-100 column against known molecular weight standards and comparing the elution volume to that of the unknown. Following this procedure resulted in an apparent mol. wt of 23,200 ± 3000 ($n = 3 \pm$ SEM) when chromatographed in a glycerol/salt buffer. This molecular weight is consistent with the data of Table 1 which suggested that GPI is a small protein.

Table 4. Purification table for crayfish glycogen phosphorylase inhibitor

Step	[Protein] (mg/ml)	Volume (ml)	Yield (%)	Fold purified[a]
Crude homogenate, (1:1), spun	25.6	2	100	1
IEF column peak fractions	0.23	13.8	55	18
Sephadex G-100 peak fraction[b]	0.02	5.2	22	210

[a]Fold purified was determined by measuring the relative amount of protein required to inhibit a defined glycogen phosphorylase activity by 50%.

[b]Native gel electrophoresis revealed two major bands which contained about 30 and 70% of the total protein. Values represent the means of two separate purifications.

DISCUSSION

The data presented in this paper indicate that a small protein is responsible for inhibiting glycogen phosphorylase activity in the hepatopancreas of the crayfish *O. virilis* and *C. bartoni*. The proteinatious nature was demonstrated by the sensitivity of the inhibitory activity to Methylene Blue, boiling, and to its precipitation at acidic pH values. The protein is apparently highly hydrophobic as it binds strongly to dye–agarose matrices, phenyl sepharose-4B and Sephadex matrices. Thus, chromatography of GPI on Sephadex columns requires the addition of 30% glycerol and 100 mM KCl in all buffers to negate the GPI–matrix interactions. However, chromatography on Blue-Dextran or phenyl sepharose results in greater than 95% loss of activity under all regimens. The highly hydrophobic nature of the enzyme may also partially account for its insensitivity to protease treatment; hydrophobic protein residues would not be readily accessible to proteases. The small size of the GPI protein was confirmed by gel exclusion chromatography on Sephadex G-100. A M_r of 23,000 was determined by comparing elution volumes of known protein standards with GPI; all elutions were performed in the presence of 30% glycerol and 100 mM KCl to reduce any protein–matrix interactions.

The GPI protein apparently acts by reversibly binding glycogen and glycogen phosphorylase (Fig. 2) and is different from other hepatopancreatic inhibitors in that it is not a protease (Fig. 1). GPI was found only in the hepatopancreas of the two species of crayfish examined, and not in the hepatopancreas of lobster, fresh and salt water clams, mussels or cockroaches. However, GPI inhibited glycogen phosphorylase from these animals, as well as type *a* glycogen phosphorylase from rabbit muscle (Table 1). GPI is apparently specific for glycogen phosphorylase since it does not inhibit other glycolytic enzymes.

Preliminary kinetic analysis of the inhibitory mechanism showed that the pattern of inhibition is not consistent with a single type of inhibition (Figs 2 and 3). The simplest explanation for the observed inhibition pattern involves GPI binding to two different molecules: glycogen and glycogen phosphorylase. However, since glycogen phosphorylase obeys a rapid equilibrium random BiBi model (Engers, 1969, 1970a,b), the inhibition patterns must be defined in terms of the inhibitory effect on both substrates (Cleland, 1963a,b). Thus, the observed competitive inhibition with respect to glycogen (Fig. 2A) and the non-competitive effect with respect to phosphate (Fig. 2B) indicate that GPI binds to glycogen with a binding coefficient between 5 and 10 μg/ml. This conclusion is supported by analysis of the glycogen phosphorylase kinetic mechanism in the presence of GPI; GPI binds glycogen in such a manner as to directly compete with glycogen phosphorylase for substrate. This results in a decrease in the amount of "enzyme–glycogen" complex, reducing the number of molecules capable of reacting with phosphate (non-competitive effect), and competing directly with glycogen phosphorylase (competitive effect).

The data of Table 3 also show that a secondary type of inhibition is apparent when glycogen phosphorylase is assayed in the presence of GPI. This inhibition has a lower affinity constant (K_{ii} = 99 μg/ml, Table 3), and is competitive with respect to phosphate binding. This may represent a conformational change in glycogen phosphorylase induced by GPI binding to the glycogen "phosphorylase–glycogen molecule" to give an enzyme with a lower K_m value for phosphate. Although two different inhibition constants are needed to describe the observed behaviour, the inhibition patterns are linear (Fig. 2) because of the large difference in the binding constants (from 10- to 20-fold). The kinetic patterns of inhibition remain to be verified by more detailed kinetic studies and by binding studies.

Table 4 shows that the GPI protein preparation, although considerably purified by isoelectric and gel permeation chromatography, is not yet homogeneous. We have made considerable efforts to further purify the GPI using ion exchange chromatography, hydrophobic affinity chromatography, and differential polyethylene glycol and ammonium sulfate precipitation, but so far these attempts have failed. We are encouraged, however, by the appearance of only two protein bands when the final sample is analysed by native gel electrophoresis, and are currently proceeding with experiments to obtain an homogeneous preparation.

The presence of a specific glycogen phosphorylase inhibitor in *O. virilis* hepatopancreas suggests that a fourth glycogen phosphorylase control mechanism exists in addition to the three known mechanisms (reversible phosphorylation, allosteric modification and control of total activity): the sequestering of glycogen by GPI. This previously undiscovered control mechanism leads one to speculate on it's possible function within the cell. Since GPI apparently binds to glycogen, it may be involved in glycogen storage—organizing glycogen into more compact structures—and so may be functionally similar to nucleosomes (which compact DNA). If this were true, one would expect (i) GPI to be directly involved in the regulation of glycogen storage, and (ii) it's rate of synthesis/breakdown to respond to changes in total glycogen, and consequently to different metabolic states (e.g. starvation, anoxia, exercise). These hypotheses remain to be experimentally verified.

REFERENCES

Aragon J. J., Tornheim K. and Lowenstein J. M. (1980) On a possible role of IMP in the regulation of phosphorylase activity in skeletal muscle. *FEBS Letts. suppl.* **117,** K56–K64.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72,** 248–254.

Brooks S. P. J. and Storey K. B. (1987) Subcellular enzyme binding in glycolytic control: *in vivo* studies with fish muscle. *Am. J. Physiol.* **255,** R289–R294.

Cohn P. (1980) In *Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation.* Elsevier/North Holland, New York.

Cleland W. W. (1963a) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. *Biochim. biophys. Acta* **67,** 173–187.

Cleland W. W. (1963b) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. III. Prediction of initial velocity and inhibition patterns by inspection. *Biochim. biophys. Acta* **67,** 188–196.

Dombradi V. (1981) Structural aspects of the catalytic and regulatory function of glycogen phosphorylase. *Int. J. Biochem.* **13,** 125–139.

Engers H. D., Bridger W. A. and Madsen N. B. (1969) Kinetic mechanism of phosphorylase *b*. Rates of initial velocities and of isotope exchange at equilibrium. *J. biol. Chem.* **244,** 5936–5942.

Engers H. D., Shecosky S. and Madsen N. B. (1970a) Kinetic mechanism of phosphorylase *a*. I. Initial velocity studies. *Can. J. Biochem. Cell Biol.* **48,** 746–754.

Engers H. D., Bridger W. A. and Madsen N. B. (1970b) Kinetic mechanism of phosphorylase *a*. II. Isotope exchange studies at equilibrium. *Can. J. Biochem. Cell Biol.* **48,** 755–758.

Helmerhorst E. and Stokes G. B. (1980) Microcentrifuge desalting: a rapid method for desalting small amounts of protein. *Analyt. Biochem.* **104,** 130–145.

Hers H. G. (1976) The control of glycogen metabolism in the liver. *A. Rev. Biochem.* **45,** 167–189.

Lackner R. (1985) Interference of proteases in the determination of pyruvate kinase activities in the hepatopancreas of *Helix pomatia L.* Artifact or a possible regulatory mechanism? *Comp. Biochem. Physiol.* **82B,** 279–282.

Laemlli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Nielson B. L. and Brown L. R. (1984) The basis for the colored silver-protein complex formation in stained polyacrylamide gels. *Analyt. Biochem.* **141,** 311–315.

Plowman K. M. (1972) In *Enzyme Kinetics*, pp. 56–75. McGraw-Hill, Montreal.

Rosell J., Baanante I. V. and Fernandez F. (1987) Glycogen phosphorylase in lobster hepatopancreas: action of proteases on the enzyme. *Comp. Biochem. Physiol.* **88B,** 983–987.

Storey K. B. (1985) Freeze tolerance in terrestrial frogs. *Cryo-lett.* **6,** 115–143.

Storey K. B. (1987) Tissue-specific controls on carbohydrate catabolism during anoxia in goldfish. *Physiol. Zool.* **60,** 601–607.

Storey K. B. and Storey J. M. (1984) Biochemical adaption for freezing tolerance in the wood frog, *Rana sylvatica. J. comp. Physiol. B* **155,** 29–36.

Storey J. M. and Storey K. B. (1985) Adaptations of metabolism for freeze tolerance in the gray tree frog *Hyla versicolor. Can. J. Zool.* **63,** 49–54.

Suelter C. H. (1985) In *A Practical Guide to Enzymology*, pp. 174–177. Wiley, New York.

Tsai C. S., Godin J. R. P. and Wand A. J. (1985) Dye-sensitized photooxidation of enzymes. *Biochem. J.* **225,** 203–208.

Vaandrager S. H., Van Marrevijk W. J. A. and Beenakkers A. M. T. (1987) Kinetic properties of glycogen phosphorylase *a*, *ab*, and *b* from flight muscle of the locust, *Locusta Migratoria. Insect Biochem.* **17,** 695–700.

Vesterberg O. (1971) Isoelectrofocusing of proteins. In *Methods in Enzymology*, Vol. 22 (Edited by Colowick S. P. and Kaplan N. O.), pp. 389–412. Academic Press, New York.

Wang D. H. and Scheer B. T. (1963) UDPG-glycogen transglucosylase and a natural inhibitor in crustacean tissues. *Comp. Biochem. Physiol.* **9,** 263–274.