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Allosteric effectors and trehalose protect larval *Manduca sexta* fat body glycogen phosphorylase B against thermal denaturation

José R. Meyer-Fernandes¹, Estela L. Arrese², Michael A. Wells^{*}

Department of Biochemistry and Center for Insect Science, Biological Sciences West, University of Arizona, Tucson, AZ 85721, USA

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Abstract

In this paper we assessed the ability of modulators of the activity of glycogen phosphorylase b from the fat body of larval *Manduca sexta* to stabilize the enzyme against thermal denaturation. This approach has allowed us to distinguish between modulators that stabilize the enzyme, presumably through some conformational effect, from those that do not affect thermal stability. For example, 5'-AMP and 5'-IMP are both positive modulators of the enzyme and the K_m s for AMP and IMP were similar, 0.71 and 1.09 mM, respectively. However, the V_{max} for AMP (123 nmol/mg/min) was 10 times higher than the value found for IMP (12.5 nmol/mg/min) and AMP increased the thermal stability of glycogen phosphorylase b, however IMP did not increase the enzyme's thermal stability. Indeed, IMP decreased both the allosteric activation of the enzyme by AMP and the thermal protection conferred by AMP. The allosteric inhibitors ADP and ATP, which in vertebrate phosphorylase bind to the same site as AMP, both increased the thermal stability of the enzyme, however with less efficiency than AMP. Inorganic phosphate increased thermal stability, but glycogen and amylose did not. Glycerol, at 600 mM, protected the enzyme against thermal inactivation, whereas sorbitol at the same concentration did not show any effect. Among the polyols tested, trehalose was the most effective in conferring thermal stability. In fact, in the presence of 20 mM AMP and 600 mM trehalose, 90% of the enzyme activity remained after 20 min at 60°C. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Fat body; *Manduca sexta*; Glycogen phosphorylase b; Allosteric effectors; Osmolytes

1. Introduction

In a number of insects glycogen phosphorylase has been shown to exist in two interconvertible forms: the phosphorylated a form, whose activity is largely independent of 5'-AMP, and the dephosphorylated b form, which is active only in the presence of high levels of 5'-AMP (Steele, 1982). Insect phosphorylases have been isolated and purified from different sources and, in general, the insect enzymes exhibit similar properties to

those of vertebrate phosphorylases (Childress and Sacktor, 1970; Applebaum and Schlesinger, 1973; Dombradi et al., 1985; Morishima and Sakurai, 1985; Vaandrager et al., 1986; Van Marrewijk et al., 1988; Gäde, 1991; Burkhardt and Wegener, 1994; Arrese et al., 1995). In *Manduca sexta*, phosphorylase b has been purified from adult flight muscle (Burkhardt and Wegener, 1994) and larval fat body (Arrese et al., 1995). Both enzymes exhibit similar characteristics with respect to the activator, AMP, the inhibitor ATP and the substrates, glycogen, and inorganic phosphate (P_i), although with different affinities for AMP, glycogen and P_i .

The binding sites for allosteric activators (AMP and IMP); inhibitors (ATP and ADP); and substrates (P_i and glycogen) in vertebrate glycogen phosphorylase have been well characterized (see for example Madsen, 1986; Newgard et al., 1989). No insect phosphorylases have been crystallized, so other methods must be used to assess the interaction of modulators with the enzyme. One approach is to determine the effect of the modu-

* Corresponding author. Tel.: +1-520-621-3847; fax: +1-520-621-9288.

E-mail address: mawells@u.arizona.edu (M.A. Wells).

¹ Permanent address: Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, RJ, Brasil. Recipient of a postdoctoral fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil.

² Current address: Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK, USA.

lators on the stability of the enzyme. For example, cold inactivation of vertebrate glycogen phosphorylase is effectively slowed by glycogen, AMP and ATP (Graves et al., 1965). However, there have been no reports on the effect of these compounds on the stability of any insect glycogen phosphorylase. In this report we describe the role of some allosteric effectors and trehalose on the thermal stability of glycogen phosphorylase b from the fat body of larval *M. sexta*. Butyraldehyde cross-linked phosphorylase b is more stable against heat inactivation than the native enzyme (Wang and Tu, 1969), however no previous studies on the thermal stability of native phosphorylase have appeared.

2. Materials and methods

First day wanderer *M. sexta* larvae from a colony maintained in this laboratory were used (Prasad et al., 1986). The substrates, glycogen phosphorylase b from rabbit muscle, phosphoglucomutase, glucose-6-phosphate dehydrogenase, coenzymes and trehalose were obtained from Sigma Chemical Company. DEAE-Cellulose (DE-52) was from Whatman, Q-Sepharose and 5'AMP-Sepharose 4B were from Pharmacia-LKB Biotechnology. All other chemicals were of analytical grade.

Phosphorylase b was purified as described by Arrese et al., 1995. Protein concentration was determined using the folin phenol reagent (Lowry et al., 1951) with bovine serum albumin as a standard. Glycogen phosphorylase activity was assayed in the direction of glycogen phosphorylation by coupling the production of glucose-1-phosphate to the reduction of NADP, using phosphoglucomutase and glucose-6-phosphate dehydrogenase. Standard conditions for measuring phosphorylase b activity were: 40 mM potassium phosphate buffer (pH 7.0), 5 mM imidazole, 2 mM Na-EDTA, 1.4 mM dithiothreitol, 5 mM magnesium acetate, 2 mM AMP, 4 μ M glucose-1,6-bisphosphate, 0.6 mM NADP, 2 mg/ml glycogen (free from AMP), 4 U of phosphoglucomutase, and 0.8 U of glucose-6-phosphate dehydrogenase in a final volume of 0.8 ml at 25°C. A negligible activity was detected in the absence of AMP, amounting to less than 0.1–0.5% of the activity measured in the presence of 2.5 mM AMP. All experiments were performed in triplicate, using two different enzyme samples. One unit of phosphorylase represents the formation of 1 μ mol glucose-1-P/min at 25°C.

Thermal stability was measured by incubating the enzyme at 60°C in 50 mM Tris-maleate buffer (pH 7.2), containing 5 mM EDTA, for different intervals of time. This temperature was chosen after preliminary experiments showed that the native enzyme was rapidly denatured at 60°C while the enzyme in the presence of AMP was relatively stable. The enzyme activity was

then assayed at 25°C. The influence of substrates, modulators and osmolytes on thermal stability was studied by the addition of varying amounts of these compounds to the incubation medium.

Kinetic parameters were derived from the experimental data using a non-linear regression fit to the Michaelis–Menten equation, and are the means of those obtained by fitting the data for each experiment.

3. Results and discussion

3.1. Effect of allosteric effectors on the thermal denaturation of glycogen phosphorylase b

AMP and IMP are the two main activators of vertebrate glycogen phosphorylase b (Dombradi, 1981). In insects, such as *Drosophila melanogaster* and *Locusta migratoria*, the activation of glycogen phosphorylase by IMP and the inhibition of the AMP-dependent activation of glycogen phosphorylase b by IMP have been studied (Dombradi et al., 1985; Van Marrewijk et al., 1988). However, the kinetics parameters for the activation by IMP have not been reported. Fig. 1 shows the dependence of phosphorylase b activity on the concentration of AMP or IMP. The behavior of the reaction with respect to AMP and IMP showed typical Michaelis–Menten kinetics with very similar K_m values of 0.71 mM and 1.09 mM, respectively. However, the V_{max} values are different for the two activators. The V_{max} for AMP (123 nmol/mg/min) was 10 times higher than the value found for IMP (12.5 nmol/mg/min). AMP and IMP induce different structural changes in vertebrate glycogen phosphorylase b (Busby and Radda, 1976), and the different values for V_{max} with AMP and IMP suggest that these activators also induce different conformational changes in fat body phosphorylase b.

When fat body glycogen phosphorylase b was incubated at 60°C for 5 min, more than 75% of the enzyme activity was lost (Fig. 2A). However, in the presence of 20 mM AMP more than 70% of the enzyme activity remained after 5 min at 60°C (Fig. 2A). On the other hand, 20 mM IMP did not protect the enzyme against thermal inactivation (Fig. 2B). In Fig. 3A and B it can be seen that IMP decreased the protection caused by AMP.

There are separate AMP and IMP binding sites in a vertebrate muscle phosphorylase b protomer (Dombradi, 1981). One of them (the nucleotide site) is the activator site, and the other (the nucleoside site) is the inhibitor site (Dombradi, 1981). Because AMP binds only to the activator site, it seems likely that occupancy of this site is correlated with increased thermal stability. IMP binds to both the activator and the inhibitor sites with comparable affinity (Dombradi, 1981), which may explain its weak activating effect (Fig. 1B). When IMP is bound to the inhibitor site, it does not cause thermal protection,

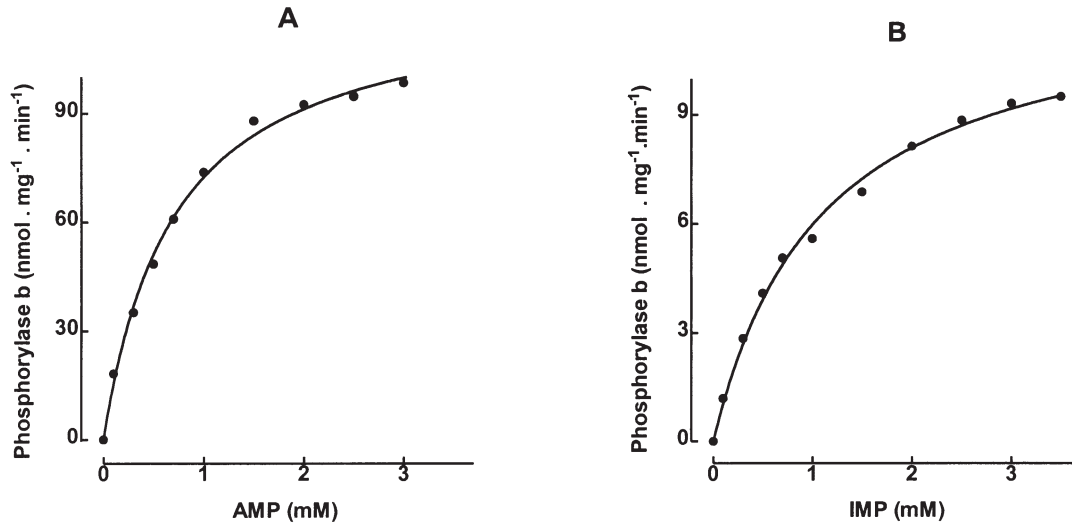


Fig. 1. The dependence of fat body glycogen phosphorylase b activity on AMP and IMP. The effect of AMP (A) and IMP (B) on the initial velocity of phosphorylase b activity. Enzyme activities were assayed as described under Materials and methods at 25°C. Values shown are the mean of triplicate determinations from two different experiments with two different enzyme preparations. The experimental data were fitted by non-linear regression to the Michaelis–Menten equation, and are means of those obtained by fitting the data of each experiment.

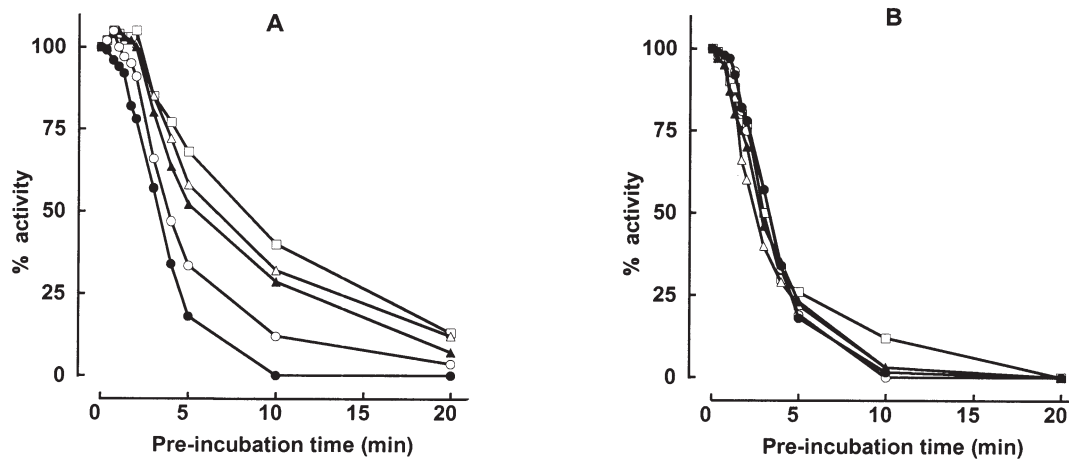


Fig. 2. The effect of AMP and IMP of the thermal stability of fat body glycogen phosphorylase b at 60°C. (A) Glycogen phosphorylase b (0.5 mg/ml) was pre-incubated for the times indicated in the absence (●), or in the presence of 1 mM AMP (○), 5 mM AMP (▲), 10 mM AMP (△), or 20 mM AMP (□). (B) Glycogen phosphorylase b (0.5 mg/ml) was pre-incubated for the times indicated in the absence (●), or in the presence of 1 mM IMP (○), 5 mM IMP (▲), 10 mM IMP (△), or 20 mM IMP (□). After pre-incubation, the samples were diluted 100-fold into the assay medium and the activity measured at 25°C. After dilution, the activity of the control (100% on the ordinate) was $98.4 \pm 5.8 \times 10^{-3}$ U/ml. Values shown are the mean of triplicate determinations from two different experiments with two different enzyme preparations. Standard errors were less than 10% in all cases and are not shown for clarity.

and by competing with AMP for the activator site, IMP decreases the thermal protection afforded by AMP (Fig. 3). The different effects of AMP and IMP on thermal protection might result from the fact that strong activators like AMP bind with the base in an *anti* orientation, whereas for weak activators like IMP this orientation is *syn* (Morange et al., 1977).

The allosteric inhibitor ATP is known to compete with AMP for binding at the activator site (Madsen, 1986). If the protection promoted by AMP is associated with occupation of the activator site, then ATP and ADP, two

potent inhibitors of fat body glycogen phosphorylase (Arrese et al., 1995), should have the same protective effect as AMP. Indeed, Fig. 4A shows that ATP and ADP are able to protect fat body glycogen phosphorylase b against thermal inactivation. However, the protective effects were not as great as with AMP and ATP was less effective than ADP. The additional phosphoryl groups present on ADP and ATP could cause different conformational changes compared to AMP. P_i was also able to increase the thermal stability of the enzyme (Fig. 4A). However, glycogen and α -amylose, which bind in the

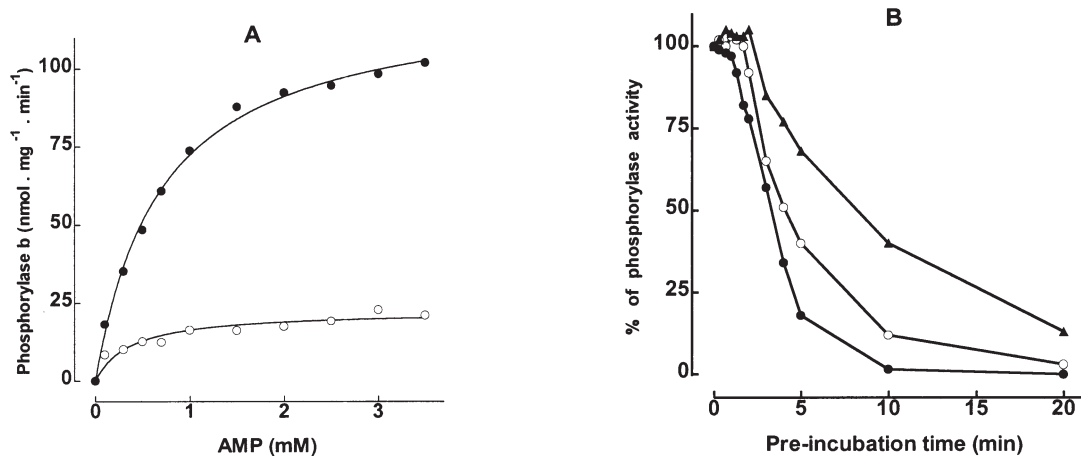


Fig. 3. The effect of IMP on fat body phosphorylase b activity and thermal stability in the presence of AMP. (A) Dependence of the glycogen phosphorylase b activity on AMP concentration in the absence (●) or in the presence of 20 mM IMP (○). (B) Thermal stability of glycogen phosphorylase b at 60°C. Glycogen phosphorylase b (0.5 mg/ml) was pre-incubated for the times indicated in the absence (●), in the presence of 20 mM AMP (▲), or 20 mM AMP plus 20 mM IMP (○). After pre-incubation, the samples were diluted 100-fold and assayed as described in the legend to Fig. 2. In (A) the experimental data were fitted by non-linear regression to the Michaelis–Menten equation, and are means of those obtained by fitting the data of each experiment.

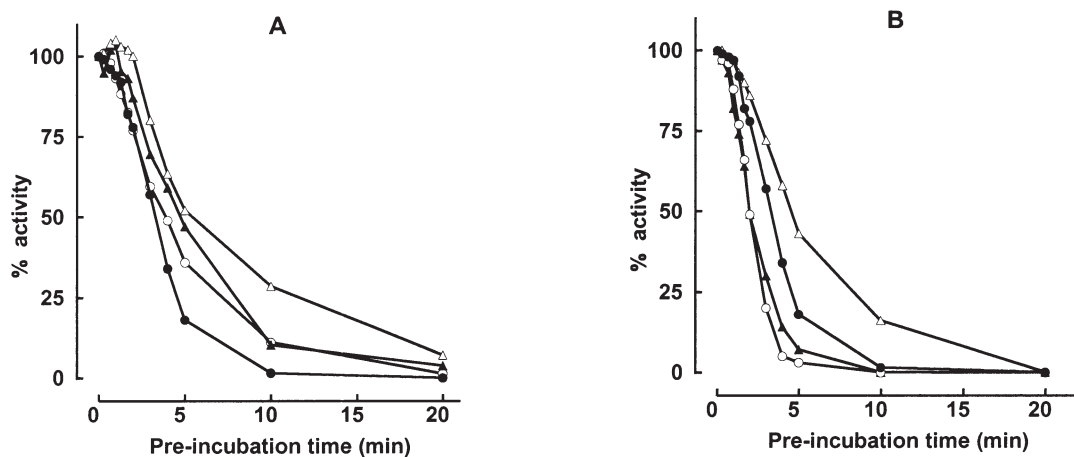


Fig. 4. The influence of allosteric effectors and substrates on thermal denaturation of fat body glycogen phosphorylase b at 60°C. (A) Glycogen phosphorylase b (0.5 mg/ml) was pre-incubated for the times indicated in the absence (●), or in the presence of 5 mM AMP (△), 5 mM ADP (▲), or 5 mM ATP (○). (B) Glycogen phosphorylase b (0.5 mg/ml) was pre-incubated for the times indicated in the absence (●), or in the presence of 30 mg/ml glycogen (○), 10 mg/ml amylose (▲), or 350 mM Pi (△). After pre-incubation, the samples were diluted 50-fold and assayed as described in the legend to Fig. 2.

glycogen-binding domain (Newgard et al., 1989), decreased the stability of the enzyme (Fig. 4B).

Many organisms and cellular systems adapt to stress conditions, such as low and high temperature, by concentrating one of several organic solutes such as sugars, polyols or methylamines (Somero, 1995; Sola-Penna and Meyer-Fernandes 1994, 1996). Among these osmolytes are glycerol and trehalose, two polyols that are present in insect hemolymph (Lum and Chino, 1990). Fig. 5A shows that 600 mM glycerol protected phosphorylase b against thermal inactivation, but sorbitol did not affect the thermal stability and manitol was less effective than glycerol in protecting the enzyme. The stabilization observed in the presence of glycerol could be attributed

to its interaction with hydrophobic centers of the enzyme; because it has been shown hydrophobic solvents stabilize phosphorylase b in an active conformation (Dreyfus et al., 1978). A regulatory hydrophobic site, distinct from the activator and glycogen-binding site has been proposed in vertebrate muscle glycogen phosphorylase b (Centeno et al., 1992). In addition, it has been proposed that glycerol favors the more folded and stable state of several proteins (Gekko and Timasheff, 1981a,b).

Trehalose, the main carbohydrate present in insect hemolymph (Lum and Chino, 1990), has also been implicated in the protection against stress conditions in other organisms (see for example Sola-Penna and

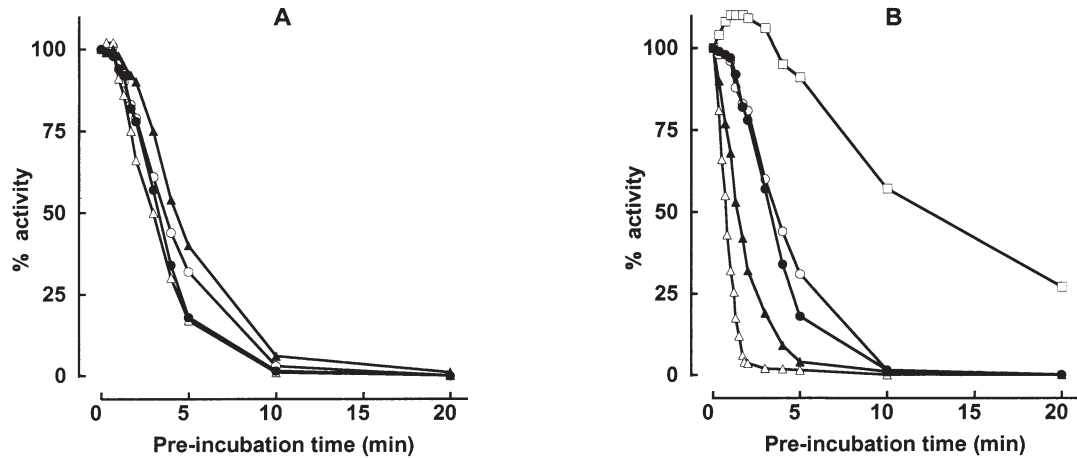


Fig. 5. The influence of carbohydrates and polyols on thermal denaturation of fat body glycogen phosphorylase b at 60°C. (A) Glycogen phosphorylase b (0.5 mg/ml) was pre-incubated for the times indicated in the absence (●), or in the presence of 600 mM sorbitol (Δ), 600 mM manitol (○), or 600 mM glycerol (▲). (B). Glycogen phosphorylase b (0.5 mg/ml) was pre-incubated for the times indicated in the absence (●), or in the presence of 600 mM sucrose (○), 600 mM maltose (▲), 600 mM lactose (△), or 600 mM trehalose (□). After pre-incubation, the samples were diluted 50-fold and assayed as described in the legend to Fig. 2. The final osmolyte concentration in the reaction medium (12 mM) did not modify glycogen phosphorylase b activity in control assays.

Meyer-Fernandes 1994, 1996; Somero, 1995). Fig. 5B shows that 600 mM trehalose protects glycogen phosphorylase b against thermal inactivation. On the other hand, sucrose did not protect the enzyme and the addition of 600 mM maltose or lactose actually decreased its thermal stability (Fig. 5B). Glycogen phosphorylase b from rabbit muscle, at the same concentration (0.5 mg/ml), was more sensitive to thermal denaturation at 60°C than fat body glycogen phosphorylase b (Fig. 6), and trehalose did not affect the thermal stability of vertebrate muscle phosphorylase b. Fig. 6A shows that the degree of protection by trehalose depended on the trehalose concentration. These data suggest that tre-

halose might interact in a specific manner with the glycogen phosphorylase b from fat body. Fig. 7 shows that AMP and trehalose act synergistically in protecting glycogen phosphorylase b from thermal denaturation. These data suggest that the protective effect of AMP and trehalose could involve different interaction sites that are only present in the insect phosphorylase.

In summary, we have shown that one can distinguish between different modulators of glycogen phosphorylase b by their ability to stabilize the protein against thermal denaturation. The differences seem to correlate with the ability of the modulator to bind to the activator site (increased thermal stability) or the nucleotide site (no

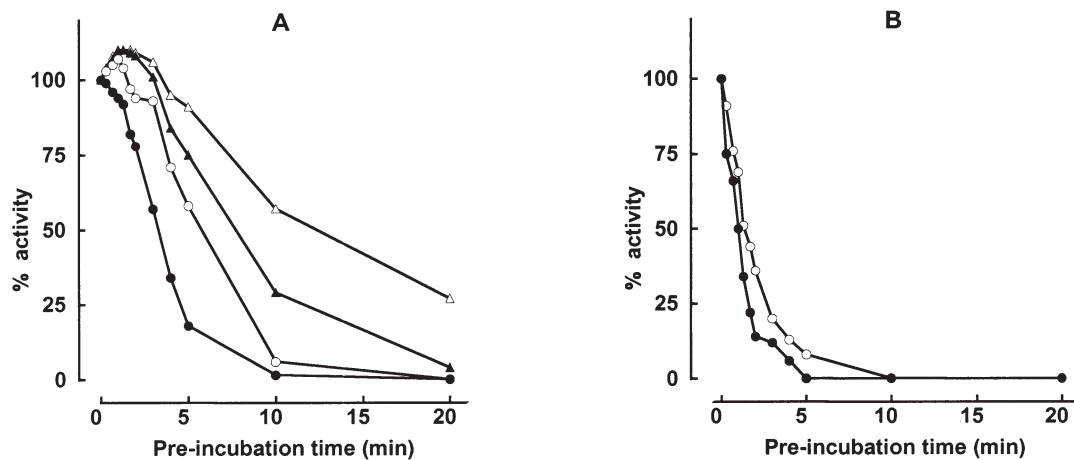


Fig. 6. The dependence of glycogen phosphorylase b thermal stability at 60°C on trehalose concentration. Fat body glycogen phosphorylase b (A) was pre-incubated for the times indicated in the absence (●), or in the presence of 200 mM trehalose (○), 400 mM trehalose (▲), or 600 mM trehalose (△). (B) Rabbit muscle glycogen phosphorylase b was pre-incubated for the same periods of time in the absence (●) or in the presence of 600 mM trehalose (○). After pre-incubation, the samples were diluted 50-fold and assayed as described in the legend to Fig. 2. After dilution the activity (100% on the ordinate) was $123.17 \pm 11.4 \times 10^{-3}$ U/ml and 20.7 ± 0.8 U/ml for the fat body and muscle glycogen phosphorylase, respectively. The final maximal trehalose concentration in the reaction medium (12 mM) did not modified activity of either enzyme.

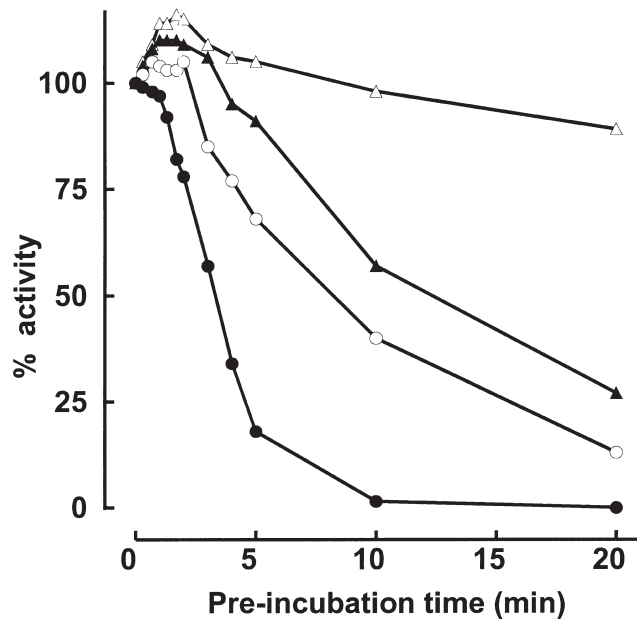


Fig. 7. Synergistic effect of AMP and trehalose on the thermal stability of fat body glycogen phosphorylase b at 60°C. Glycogen phosphorylase b (0.5 mg/ml) was pre-incubated for the times indicated in the absence (●), or in the presence of 20 mM AMP (○), 600 mM trehalose (▲) or 20 mM AMP plus 600 mM trehalose (△). After pre-incubation, the samples were diluted 50-fold and assayed as described in the legend to Fig. 2. After dilution the activity (100% on the ordinate) was $110.4 \pm 10.8 \times 10^{-3}$ U/ml. The final trehalose concentration in the reaction medium (12 mM) did not modify the glycogen phosphorylase b activity.

effect on thermal stability). In addition, we have shown that insect phosphorylase, but not vertebrate phosphorylase, is stabilized by trehalose. Because of the high concentration of trehalose required, the physiological significance of this observation is not clear at the moment, but warrants further investigation.

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