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Studies on the Effect of Temperature on the Activity and Stability of Cyanobacterial ADP-Glucose Pyrophosphorylase

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The effect of temperature on the activity and stability of ADPglucose pyrophosphorylase from *Anabaena* PCC 7120 was studied. Experimental optima temperatures were found around 37–40°C or 42–45°C, depending on the absence or the presence of allosteric effectors in the assay medium, respectively. In the range of temperature where the enzyme is stable, curved Arrhenius plots were obtained, indicating a transition temperature between 9 and 12°C. Since these results were observed for both the forward and reverse reaction, with two different sets of substrates and two entirely different assay procedures, it seems unlikely that the effect can be on any component of the system other than the enzyme itself. Results suggest that cyanobacterial ADPglucose pyrophosphorylase undergoes conformational changes at different temperatures, rendering structures with different catalytic efficiencies. The different structures of the enzyme were visualized by emission fluorescence. ADPglucose pyrophosphorylase was irreversibly inactivated when exposed to temperatures above 40°C. Inactivation was dependent on temperature and followed first order kinetics. The substrate, ATP, and the allosteric effectors, 3PGA and Pi, effectively protected the enzyme against thermal inactivation. Protection afforded by ATP was affected by $MgCl_2$. These results suggest that the binding of the effectors to the enzyme resulted in conformational changes of the protein, rendering structures more stable to temperature treatments. Similar structures could be adopted by the enzyme in different environments, since the higher stability was observed in media containing either high ionic strength or high hydrophobicity.

Key Words: **ADP-glucose pyrophosphorylase; glycogen/starch biosynthesis; *Anabaena*.**

ADPglucose pyrophosphorylase (ADPGlc PPase, EC 2.7.7.27) catalyzes the reversible synthesis of ADPGlc and PPi from ATP and Glc1P in the presence of a divalent metal ion. The enzyme plays a key role in plants and bacteria, since it mediates the regulatory step in the biosynthesis of reserve polysaccharides, starch and glycogen, respectively (1–4). Usually, the catalytic activity of the enzyme is allosterically regulated by effectors derived from the dominant carbon assimilation route in the organism (2, 4).

Numerous studies have been carried out on ADPGlc PPase from different sources in order to establish structure to function relationships. It is now clear that there are significant differences in allosteric regulation as well as in structure between the plant and bacterial protein (1, 2). The enzyme from bacteria is a homotetramer of 200-kDa molecular mass, inhibited (depending on the source) by AMP and ADP and activated by different glycolytic intermediates, such as fructose-1,6bisP, fructose-6P, or pyruvate (1–3, 5). In higher plants and green algae, ADPGlc PPase is mainly regulated by 3PGA (activator) and Pi (inhibitor), the enzyme being composed by two different subunits, α and β, that give rise to an $\alpha_2\beta_2$ heterotetramer (1, 2, 4, 6, 7). Interestingly, ADPGlc PPase from cyanobacteria exhibits intermediate properties, being homotetrameric in structure but regulated by 3PGA and Pi and immunologically more related with the plant enzyme (2).

Besides these differences, purification of ADPGlc PPase from bacteria and plants usually involves a heat treatment step, the enzyme remaining active after 5 min incubation at 60–65°C. Only a few exceptions

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were reported indicating a heat inactivation of ADPGlc PPase from *Chlamydomonas reinhardtii* (7), as well as of mutant enzymes from potato tuber (8), bacteria (9), and cyanobacteria (10). Despite the importance and utility of the heat treatment for the purification of ADPGlc PPase, this step is performed empirically. Studies concerning the thermal effect on the kinetics of the enzyme from the bacterium *Rhodospirillum rubrum* were previously reported (11) and a work dealing with the enhanced stability exhibited by mutants of the maize endosperm enzyme was made by Greene and Hannah (12). More recently, Ballicora *et al.* (13) analyzed the role of Cys 12 in the small subunit of ADPGlc PPase for the heat stability of the enzyme from higher plants. However, a complete study on the effect of temperature on the activity and stability of the enzyme from other sources is lacking at the present time. In this work we report such a characterization mainly performed utilizing ADPGlc PPase from the cyanobacterium *Anabaena* PCC 7120.

MATERIALS AND METHODS

Chemicals. [^{14}C]glucose-1P and [^{32}P]PPi were purchased from Dupont-New England Nuclear. ADPGlc and 3PGA were from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest available commercial grade.

Enzyme isolation and protein determination. Cyanobacterial ADPGlc PPases used in this study were from *Anabaena* PCC 7120 or the recombinant enzyme from *Escherichia coli* B, strain AC70R1-504, cells transformed with pAnaE3b (10, 14). Highly purified enzyme was obtained by the procedure described by Iglesias *et al.* (15).

Protein was determined by the modified Lowry method (16), using bovine serum albumin as the standard.

Assay of ADPGlc PPase. Activity of ADPGlc PPase was assayed at the specified temperatures either, in pyrophosphorolysis (Assay A) or in ADPGlc synthesis (Assay B) direction, essentially as described by Ghosh and Preiss (17).

Assay A. Pyrophosphorolysis of ADPGlc was followed by the formation of [^{32}P]ATP in the presence of [^{32}P]PPi. The reaction mixture contained 20 μmol Mops-KOH buffer, pH 7.5, 2 μmol $MgCl_2$, 0.5 μmol ADPGlc, 0.5 μmol [^{32}P]PPi (ca. 3000 cpm/nmol), 50 μg BSA, 2.5 μmol NaF, and enzyme in a total volume of 0.25 ml. The reaction was stopped by adding 3 ml of cold 5% TCA. The [^{32}P]ATP formed was measured as in (17).

Assay B. The synthesis of ADP[^{14}C]Glc from [^{14}C]Glc1P and ATP was measured in 0.2 ml final volume of a reaction mixture containing 16 μmol Mops–KOH buffer, pH 7.5, 50 μg BSA, 1.5 μmol $MgCl_2$, 0.5 μmol ATP, 0.1 μmol α-[^{14}C]Glc1P (about 1000 cpm/nmol) and 0.15 unit of inorganic pyrophosphatase in a final volume of 0.2 ml. Assays were initiated by addition of enzyme and terminated by heating in a boiling water bath for 30 s. Radioactive ADPGlc formed was measured as previously described (17).

The above described assays (A and B) correspond to measurements under nonactivated conditions. Assays of the activated enzyme were carried out at identical conditions except for the addition of 2.5 mM 3PGA in the corresponding mixture. One unit of ADPGlc PPase activity is defined as that amount of enzyme catalyzing synthesis of 1 μmol of product per minute under the stated reaction conditions.

Thermal inactivation of the enzyme. The purified enzyme (about 0.6 mg/ml) was incubated in a medium containing 50 mM Mops–KOH, pH 7.5 at the different temperatures and with the additions specified for each condition. After the corresponding specified time, thermal treatment was stopped by addition of cold buffer (10-fold volumes) and by maintaining the enzyme at 4°C until assay of activity, which occurred within 5 min after thermal treatment; the latter conditions caused no effect on the enzyme activity.

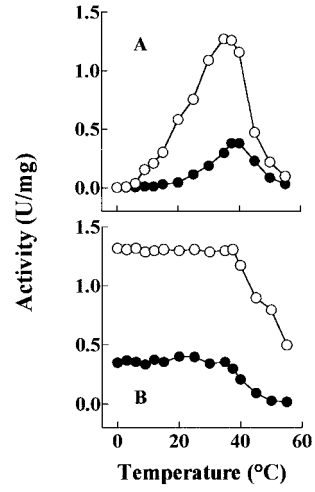

FIG. 1. Effect of temperature on the activity and stability of cyanobacterial ADPGlc PPase. (A) Purified enzyme was assayed in the ADPGlc synthesis direction at the stated temperatures in the absence (empty circles) or in the presence (filled circles) of 0.1 mM Pi. (B) The effect of temperature on the enzyme stability was checked by incubating the enzyme at the different stated temperatures for 10 min and then assaying, at 37°C, for ADPGlc synthesis activity, in the absence (empty circles) or in the presence (filled circles) of 0.1 mM Pi in the precincubated samples.

Treatment of kinetic data. All velocity data were obtained under linearity conditions respect to time and protein concentration. Mops buffer was selected to carry out these studied because the low dependence of pH with temperature (-0.011 ΔpH/Δtemperature, see Ref. 18) and pH of the solutions were adjusted to the corresponding work temperature. Values of $S_{0.5}$, $A_{0.5}$, and $I_{0.5}$ (corresponding to the concentrations giving 50% maximal activity, activation, and inhibition, respectively), Hill coefficients (n_H), and V_{max} were calculated using the computer program developed by Brooks (19). All kinetic parameters are the mean of at least two determinations and are reproducible to within $\pm$10%.

Fluorescence studies. Fluorescence measurements were performed in a Kontron SFM spectrofluorometer using an excitation wavelength of 295 nm. The emission spectra were recorded in the 420- to 300-nm range at 5 and 30°C. Each spectrum was recorded in duplicate. Emission and excitation band widths were 10 nm. Before each assay, samples containing 20 μg/ml of pure enzyme diluted in buffer 50 mM Mops–KOH, pH 7.5 (with eventual addition of 3PGA and/or Pi), were filtered through a Millipore 0.22-μm filter membrane to avoid light scattering.

RESULTS

Figure 1 shows the effect of temperature on the activity and stability of ADPGlc PPase from *Anabaena* PCC 7120. When the enzyme was assayed in the ADPGlc synthesis direction at different temperatures, either in the absence or in the presence of the allosteric inhibitor Pi, experimental maxima temperatures in the

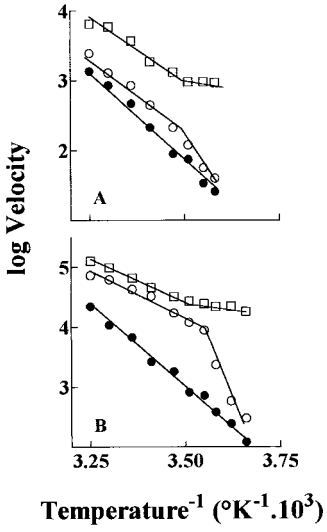

FIG. 2. Arrhenius plots (20, 21) for the activity of cyanobacterial ADPGlc PPase assayed at different temperatures. Activity was assayed in the ADPGlc synthesis (A) or in the pyrophosphorolysis (B) direction. Assays were carried out at the stated temperatures without additions (empty circles), with 2.5 mM 3PGA (empty squares), or plus 0.1 mM Pi (filled circles), in the assay medium.

range of 37–40°C or 42–45°C were, respectively, obtained (Fig. 1A). Assays carried out in the presence of 3PGA (allosteric activator), instead of Pi, gave essentially the same optimum temperature (42–45°C) (data not shown). Figure 1B shows that when the enzyme was preincubated during 10 min at the different temperatures and then assayed at 37°C, a decrease of activity for samples maintained at preincubation temperatures higher than 40°C was observed, either for activity assays performed in the absence or in the presence of Pi (or 3PGA). These data suggest that, in the absence of allosteric effectors, the enzyme is unstable at temperatures higher than 40°C, and that the allosteric effectors partially protect against temperature inactivation. Essentially the same results were obtained when enzyme activity was assayed in the pyrophosphorolysis direction.

Arrhenius plots (20, 21) of the data of ADPGlc PPase activity assayed at different temperatures (in the range 0–40°C) and in the absence or in the presence of allosteric effectors (Pi and/or 3PGA) are shown in Fig. 2. In both assay directions, ADPGlc synthesis (Fig. 2A) and pyrophosphorolysis (Fig. 2B), downwardly curved plots were obtained for assays performed in the absence of allosteric effector, with two straight lines meeting at a transition temperature in the range 9–12°C. The presence of Pi alone in the enzyme assay medium abolished curvatures and rendered a single straight line for the plot (Figs. 2A and 2B). On the other hand, when enzyme assays were carried out in the presence of 3PGA, Arrhenius plots upwardly curved with a transition temperature also in the range 9–12°C, were obtained, as shown in Figs. 2A and 2B. A similar result, respect to upwardly curved Arrhenius plots was obtained when both, 3PGA and Pi were present in the medium (data not shown). In all of the conditions where two straight lines for Arrhenius plots were obtained (without additions and assays in the presence of 3PGA or 3PGA and Pi) the break-point for the change in the curve occurred at the same range of temperature.

One possible cause of the occurrence of curved Arrhenius plots may be attributed to an effect of temperature on any or all of the components of the reaction (20). However, this can be discarded for the case of cyanobacterial ADPGlc PPase. Studies on the effect of pH on the reaction of ADPGlc synthesis showed the same pH optimum value of 7.5 at temperatures of 5 or 30°C and whether 3PGA was absent or present in the assay medium (data not shown). Another explanation for our results of Arrhenius plots is the existence of two or more conformational forms of the enzyme with distinctive kinetic and regulatory properties, occurring at different temperatures: below and over 9–12°C (20). Results that follow suggest this possibility.

The effect of temperature on cyanobacterial ADPGlc PPase activity illustrated in Fig. 2 indicates how V_{max} of the enzyme is affected. Experiments carried out at substrate or effector (3PGA) concentrations three-fold higher show the same patterns as shown in Figs. 2A and 2B (data not shown). Enthalpy of activation values for the different experimental conditions, calculated from Arrhenius plots (20, 21), are summarized in Table I.

It is worth to point out that the above just specified does not mean that affinity of ADPGlc PPase for substrates and effectors was completely independent of temperature. On the contrary, distinctive changes

TABLE I

Enthalpy of Activation for the Reaction Catalyzed by ADPGlc PPase (Pyrophosphorolysis and Synthesis Direction) Calculated from Arrhenius Plots Obtained under the Different Specified Experimental Conditions

	ΔHa (cal/mol)	
Assay	0–10°C	10–40°C
ADPGlc synthesis		
No addition	30500 ± 3000	19600 ± 1100
+ 3PGA 2.5 mM	5500 ± 1600	15300 ± 1900
+ Pi 2 mM	24300 ± 800	24300 ± 800
+ Pi + 3PGA	9300 ± 1600	18700 ± 1100
Pyrophosphorolysis		
No addition	58800 ± 6400	14100 ± 1800
+ 3PGA 2.5 mM	4500 ± 900	11400 ± 400
+ Pi 2 mM	24400 ± 800	24400 ± 800
+ Pi + 3PGA	11900 ± 1200	22200 ± 2400

TABLE II

Kinetic Behavior of Purified ADPGlc PPase at 5 and 30°C

Substrate	3PGA 2.5 mM	Kinetic parameter			
		5°C $S_{0.5}$ (mM)	n_H	30°C $S_{0.5}$ (mM)	n_H
Pyrophosphorolysis					
PPi	−	0.27 ± 0.02	1.0	0.066 ± 0.005	2.0
	+	0.074 ± 0.005	1.0	0.064 ± 0.006	1.3
ADPGlc	−	0.080 ± 0.007	0.7	1.06 ± 0.04	2.0
	+	0.052 ± 0.003	0.7	0.72 ± 0.05	1.5
ADPGlc synthesis					
Glc1P	−	0.55 ± 0.04	3.0	0.17 ± 0.02	1.0
	+	0.044 ± 0.003	1.2	0.078 ± 0.008	1.7
ATP	−	0.11 ± 0.01	2.0	0.61 ± 0.05	1.8
	+	0.082 ± 0.007	1.7	0.30 ± 0.03	1.1

were observed in saturation kinetics for the ligands of the enzyme when determined at two different temperatures: 5 and 30°C (below and above the observed break point). Table II shows $S_{0.5}$ and n_H values for the substrates of the enzyme, determined at 5 and 30°C and in the absence or in the presence of 3PGA. Except for PPi at 30°C (where no change was observed), 3PGA reduced $S_{0.5}$ values for the substrates in all the conditions analyzed (Table II). In the pyrophosphorolysis direction, saturation kinetics for the substrates were hyperbolic at 5°C and exhibited cooperativity at 30°C, with the sigmoidicity diminished by 3PGA (Table II). In contrast, in the ADPGlc synthesis direction, cooperativity was higher at the lower temperature (Table II). In the absence of the activator, $S_{0.5}$ values for PPi and Glc1P were higher at the lower temperature, whereas in the presence of 3PGA the values at 5 and 30°C were less affected (Table II). On the other hand, values of $S_{0.5}$ for ADPGlc and ATP increased at higher temperature and independent of the presence of 3PGA in the medium (Table II), thus showing that the apparent affinity of these ligands with the enzyme is higher at low temperatures.

On the other hand, V_{max} for ADPGlc PPase was affected by temperature and 3PGA in a different way, depending if the reaction is performed in the pyrophosphorolysis or the ADPGlc synthesis direction. Table III shows the ratio of the respective V_{max} (pyrophosphorolysis over synthesis), determined at 5 or 30°C and in the absence or in the presence of 2.5 mM 3PGA in the assay medium. As shown, the ratio is between 2.3 and 2.8 in the different conditions, except at 30°C and in the absence of 3PGA, where V_{max} for the reaction of pyrophosphorolysis was relatively higher. As also observed in Table III, at 30°C 3PGA behaved as a better activator in the ADPGlc synthesis direction than in pyrophosphorolysis, since at this temperature the allosteric activator restored the ratio of V_{max} to a value of 2.6.

Allosteric regulation of cyanobacterial ADPGlc PPase by the effectors 3PGA and Pi exhibited a temperature sensitivity, as illustrated in Figs. 3A and 3B.

TABLE III

Ratio of Maximal Catalytic Activity (Pyrophosphorolysis over Synthesis) of ADPGlc PPase as Affected by Temperature and the Allosteric Activator

Condition		V_{max} ratio (pyroph./synth.)
5°C	− 3PGA	2.3
	+ 3PGA	2.8
30°C	− 3PGA	6.5
	+ 3PGA	2.6

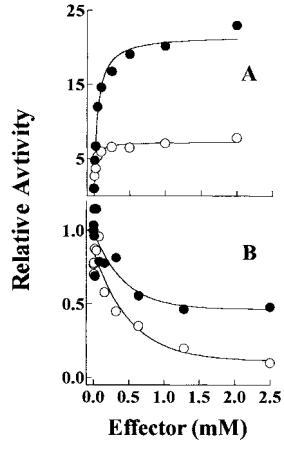

FIG. 3. Allosteric regulation of ADPGlc PPase at two different temperatures. The enzyme was assayed in the ADPGlc synthesis direction in the presence of different concentrations of 3PGA (A) or Pi (B) and at 5°C (filled circles) or 30°C (empty circles). Relative activity is calculated respect to the value obtained in the absence of effectors at the corresponding temperature: 0.22 and 5.1 U · mg^{-1} at 5 and 30°C, respectively.

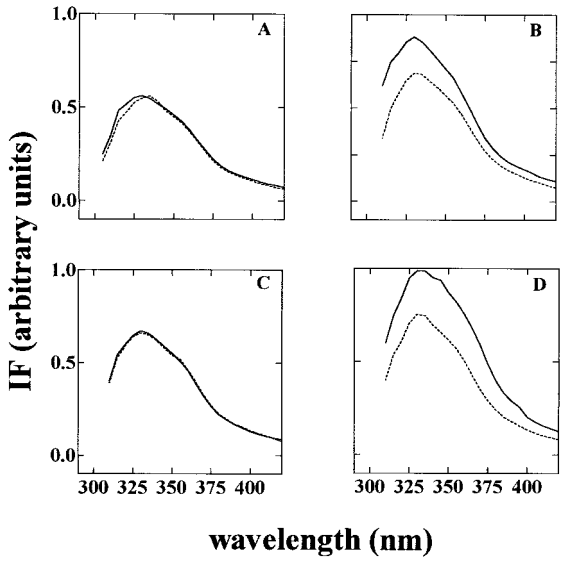

FIG. 4. Intrinsic tryptophan emission spectra of AGPGlc PPase in different conditions. Spectra of the purified enzyme in 50 mM Mops–KOH, pH 7.5, without further additions (A), plus 2.5 mM 3PGA (B), plus 1 mM Pi (C), or plus 2.5 mM 3PGA and 1 mM Pi (D) were performed at 5°C (full line) or at 30°C (dashed line). IF, intensity of fluorescence.

When studied in the ADPGlc synthesis direction, saturation kinetics for the allosteric activator 3PGA showed differences (mainly respect to activation fold) when assayed at 5 or 30°C (Fig. 3A). Thus, at 5°C ADPGlc PPase was activated 23-fold by 3PGA with an $A_{0.5}$ of 0.062 ± 0.004 mM, whereas at 30°C maximal activation was of 7.9-fold and the $A_{0.5}$ was 0.031 ± 0.001 mM (Fig. 3A). At both temperatures, the saturation curves were hyperbolic (n_H of 1.0). Enzyme inhibition by Pi was lower at the lower temperature, with an $I_{0.5}$ of 1.5 ± 0.2 mM at 5°C, compared with the $I_{0.5}$ value of 0.18 ± 0.02 mM obtained at 30°C (Fig. 3B).

The temperature-dependent different kinetic and regulatory properties exhibited by ADPGlc PPase (including the nonlinear Arrhenius plots) may suggest the existence of different forms of the enzyme occurring at 5 or 30°C. Thus, emission fluorescence spectra of tryptophan residues in the purified enzyme (excitation at 295 nm, see Ref. 22) were determined under the different experimental conditions (5 and 30°C, in the absence or in the presence of allosteric effectors). Peaks for maximal emission of the enzyme alone were found at 330 or 335 nm, for spectra recorded at 5 or 30°C, respectively (Fig. 4A). Although the fluorescence emissions in Fig. 4A were quite broad, these data suggest that at the lower temperature, tryptophan residues in the enzyme may be surrounded by a more hydrophobic environment (22). This was reinforced by results showing that in the presence of 3PGA spectral behaviour of the enzyme exhibited a similar maximal emission peak (around 330 nm) at both temperatures, but intensity in the intrinsic fluorescence was higher at 5°C than at 30°C (Fig. 4B), also indicating the shift to a nonpolar environment at the lower temperature (22). Interestingly, and in agreement with linear Arrhenius plot (see Fig. 2), in the presence of Pi the emission spectrum showed no difference with temperature (Fig. 4C); whereas when both allosteric effectors were present in the medium, intensity of the enzyme's intrinsic fluorescence was about 25% higher at 5°C than to at 30°C (Fig. 4D).

As suggested by results described in Fig. 1, ADPGlc PPase from cyanobacteria is unstable when exposed at temperatures above 40°C, in the absence of allosteric effectors. Figure 5 shows that the enzyme is irreversibly inactivated by incubation at temperatures of 48°C and above. Inactivation is temperature- and time-dependent following first order kinetics. Inactivation half times of 68, 41, and 25 s were obtained for incubations at 48, 54, and 60°C, respectively (Fig. 5). The rate constant (k) of the inactivation process was calculated from the slope of Fig. 5, and the inactivation enthalpy (ΔH_i) was estimated to be 4.33 kcal/mol, from a plot of $\ln(k/T)$ against $1/T$ (inset Fig. 5), using the Eyring absolute rate equation (23):

$$\ln(k/T) = -\Delta H_i/RT + \Delta S_i/R + \ln(A), \qquad [1]$$

where T is the absolute temperature, R is the gas constant, ΔS_i is the inactivation entropy, and A is the ratio of Boltzmann's constant and Planck's constant.

The effect of different compounds related with ADPGlc PPase (substrates and allosteric effectors) on the thermal inactivation of the cyanobacterial enzyme at 60°C is shown in Table IV. As illustrated, Pi and 3PGA afforded effective protection as indicated by increase in the time required for half-inactivation of the enzyme

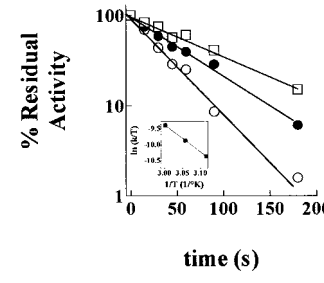

FIG. 5. Irreversible thermal inactivation of cyanobacterial ADPGlc PPase. The enzyme was incubated as described under Materials and Methods at 48°C (empty squares), 54°C (filled circles), or 60°C (empty circles). At the stated times, thermal treatment was stopped by addition of cold buffer (10-fold volumes) and by maintaining the enzyme at 4°C until assay of activity, which occurred within 5 min after thermal treatment. Inset: Estimation of the inactivation enthalpy according to (23).

TABLE IV

Effect of Different Metabolites on the Thermal Inactivation of ADPGlc PPase at 60°C

Addition	Concentration	$t_{0.5}$ (s)
None		25
Glc1P	1mM	46
	5mM	56
ADPGlc	1mM	31
	5mM	51
Mg^{++}	5mM	30
PPi	5mM	91
ATP	1mM	451
	5mM	>475
3PGA	1mM	388
	5mM	>650
Pi	1mM	267
	5mM	>531

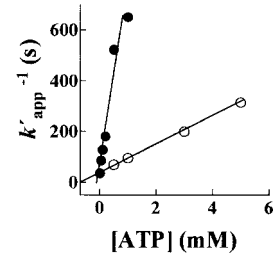

FIG. 6. Dissociation constant (K_d) for the interaction ADPGlc PPase–ATP calculated by the protection afforded by the substrate against thermal inactivation of the protein. The enzyme was incubated at 60°C in the absence or in the presence of the stated concentrations of ATP alone (filled circles) or plus 5 mM $MgCl_2$ (empty circles). At different times (0, 30, 60, and 120 s) aliquots were withdrawn and the remaining activity assayed. Inactivation kinetic data obtained at the different conditions were plotted as described in the text.

(Table IV). Neither glucose-1P, nor ADPGlc, nor Mg^{2+} significatively modified thermal inactivation of the PPase, whereas PPi slightly increased the $t_{0.5}$ value (Table IV). ATP behaved as a protective ligand (Table IV), with other nucleotides (ADP, AMP) exhibiting a significant lower protective effect (not shown). Of interest is the effect of Mg^{2+} on the protection afforded by ATP; although Mg^{2+} alone had no effect on thermal inactivation (Table IV), it reduced the protective effect exhibited by a determined concentration of nucleotide (see below).

Protective effects of ATP, Pi and 3PGA were dependent on substrate or allosteric effector concentration in the activation medium, with practically total protection observed at relatively high levels (5 mM) of the corresponding effector. This fact was used to calculate the dissociation constants (K_d) for the binding of the corresponding ligand to ADPGlc PPase. In this way, protection against inactivation of the enzyme at 60°C afforded by different concentrations of ATP, Pi or 3PGA, either in the absence or in the presence of 5 mM $MgCl_2$ was studied. Inactivation kinetic data obtained at the different conditions were plotted according to Mildvan and Leigh (24) by using the equation:

$$1/k'_{app} = 1/k' + a/K_d \cdot k', \qquad [2]$$

where k' is the observed first-order rate constant of inactivation in the absence of the protective agent, and k'_{app} the apparent inactivation constant obtained at different concentrations of a, the compound affording protection. Figure 6 shows plots of the reciprocal of k'_{app} obtained for thermal inactivation carried out at different ATP concentrations in the absence or in the presence of $MgCl_2$. As shown, these plots gave straight lines from which K_d values of 0.040 mM and 0.71 mM were calculated for the binding of ATP to the enzyme in the absence or in the presence of 5 mM $MgCl_2$, respectively (Table V). These results indicate that the binding of the complex Mg-ATP to the enzyme (which protects against thermal inactivation) occurs with lower affinity than the binding of ATP alone.

In a similar fashion, from the protection afforded by different concentrations of Pi or 3PGA against thermal inactivation of ADPGlc PPase from *Anabaena* PCC 7120, K_d values for the binding of these effectors to the enzyme were calculated (Table V). As shown, interactions of Pi and 3PGA with the enzyme were not affected by the presence of $MgCl_2$ in the incubation media.

Velocity for thermal inactivation of ADPGlc PPase was dependent on the composition of the medium where thermal treatment was carried out. Table VI shows that in incubation media containing PEG or KCl the enzyme was more stable to heat treatment, with $t_{0.5}$ values for inactivation increasing between 3- and 13-fold, depending on the compound and its concentration in the incubation medium. When results in Table VI are analyzed in terms of molarity, it is clear that PEG afforded the more significant protection to the

TABLE V

Dissociation Constants for the Interaction with ADPGlc PPase, Calculated from the Protection against Thermal Inactivation Afforded by Different Concentrations of the Corresponding Ligands

Compound		K_d (mM)
ATP		0.040 ± 0.003
	+ 5 mM Mg^{++}	0.71 ± 0.05
3PGA		0.081 ± 0.006
	+5 mM Mg^{++}	0.090 ± 0.008
Pi		0.10 ± 0.01
	+ 5 mM Mg^{++}	0.10 ± 0.01

TABLE VI

Effect of Different Compounds Present in the Medium for Inactivation of ADPGlc PPase at 60°C

Addition		$t_{0.5}$ (s)
None		25
Sucrose	48% (w/v)	34
	64% (w/v)	84
Glycerol	35% (w/v)	21
	70% (w/v)	36
KCl	0.2 M	72
	1.6 M	154
PEG (8000)	28% (w/v)	146
	56% (w/v)	332

enzyme against thermal inactivation. On the other side, glycerol was a poor protective agent of the enzyme against inactivation and sucrose exhibited significant protection only at high concentration (Table VI).

DISCUSSION

Activity of cyanobacterial ADPGlc PPase showed (under the experimental conditions utilized in this work) optima temperatures around 37–40°C or 42–45°C depending on the absence or the presence of allosteric effector (Pi and 3PGA) in the assay medium, respectively. Above these temperatures the enzyme was unstable, with significant loss of activity within the time period for the assay. In the range of temperature where the enzyme is stable, curved Arrhenius plots were obtained, for assays performed in the absence of allosteric regulators or in the presence of the activator 3PGA (alone or together with the inhibitor Pi). The curved Arrhenius plots most likely result from two straight lines that meet at a transition temperature in the range 9–12°C; as a consequence of conformational changes undergone by ADPGlc PPase, rendering two forms of the enzyme with different energy of activation. It is worth to point out that in the presence of 3PGA (plus or minus Pi) discontinuities in Arrhenius plots occur with the particularity that higher activation energies taking place at the higher temperatures. This type of plot, with an upward bend, has been reported as quite unusual (20).

Similar type of Arrhenius plots at the different assay conditions were observed for both, the forward and the reverse reaction catalyzed by ADPGlc PPase. The fact that a similar behavior of the enzyme activity as a function of temperature was observed with two different sets of substrates and two entirely different assay procedures, strongly supports that the effect of temperature is on the enzyme itself (11, 20). Results suggest that cyanobacterial ADPGlc PPase undergoes conformational changes at different temperatures, rendering structures that possess: (i) different catalytic efficiencies, (ii) altered capability to catalyze the forward or reverse reaction (ADPGlc synthesis or pyrophosphorolysis), and (iii) different sensitivity to allosteric regulators. Thus, the enzyme's form existing at low temperature exhibits lower apparent affinities toward its substrates, is more sensitive to activation by 3PGA and less inhibited by Pi. Allosteric regulators of the enzyme induces similar changes in the structure of the protein and the interaction with the enzyme is also dependent on the temperature. An early report on ADPGlc PPase from *R. rubrum* also showed the occurrence of different forms of the enzyme with temperature (11). However, the behavior of the *R. rubrum* enzyme was different (respect to transition temperatures and number and properties of the forms) compared with the present study of the cyanobacterial PPase.

The occurrence of structural conformational changes in ADPGlc PPase with temperature was reinforced by studies on emission fluorescence of tryptophan residues of the protein. Results showed that, at 5°C these residues locate in a more non-polar environment (respect to what occurs at 30°C) since: (i) maximum emission peak of the enzyme shifts 5 nm to the UV-region (enzyme alone) or (ii) intrinsic emission fluorescence is higher in intensity (enzyme plus 3PGA or plus 3PGA and Pi) (24). Interestingly this change in the intensity of emission fluorescence with temperature was not observed when the enzyme was incubated in the presence of Pi alone, in agreement with kinetic studies showing linear Arrhenius plots in this experimental condition. Fluorescence spectroscopy also support the occurrence of conformational changes in the enzyme induced by the allosteric effectors. Thus, in the presence of 3PGA intrinsic fluorescence of the enzyme was found higher, specially at 5°C.

ADPGlc PPase purified from *Anabaena* PCC 7120 exhibited instability when exposed to temperatures above 40°C in buffered aqueous media. Under these conditions the enzyme was irreversibly inactivated. Inactivation was dependent on temperature and followed first order kinetics. The substrate, ATP, and the allosteric effectors, 3PGA and Pi, effectively protected the enzyme against thermal inactivation. Protection by Pi to thermal inactivation is relevant and most probably is the basis of the use of heat treatment in the purification of cyanobacterial ADPGlc PPase (15). Protection afforded by ATP was affected by $MgCl_2$. These results suggest that the binding of the effectors to the enzyme resulted in conformational changes of the protein, rendering structures more stable to temperature treatments, by probably favoring the formation of higher number of hydrogen bonds and the arrangement of ion pairs (25).

The protective effect of the ATP, 3PGA, and Pi on thermal inactivation of ADPGlc PPase allowed us to determine dissociation constants for the binding of the substrate and the allosteric effectors to the enzyme. Thus, by using the approach developed by Mildvan and

Leigh (24) we calculate K_d values for ATP of 0.040 and 0.71 mM, depending on the absence or the presence of $MgCl_2$, respectively. In a similar way, K_d values for 3PGA and Pi were, respectively, determined as 0.081 and 0.10 mM. Parameters for the interaction between substrates and effectors of ADPGlc PPase from cyanobacteria (15) and other sources (1, 3, 4, 5, 7–10) were previously determined under conditions of kinetic assays. In this way, here we report a different estimation of the binding parameter, by measuring dissociation constants in the absence of other effectors or substrates of the enzyme.

The stability of cyanobacterial ADPGlc PPase to thermal treatment was also increased in media containing high concentrations of KCl or PEG. The effect of these compounds can be understood from reports indicating that the binding of metal ions to proteins, the formation of new ion pairs, and increases in hydrophobicity constitute features identified as factors that enhance enzymes thermostability (26). The protective effect provoked by conditions of high ionic strength has been reported in a number of other enzymes. It is known that elevated concentrations of different salts (including those of monovalent cations) induce a general stabilizing effect on the native structure of globular proteins by inducing their preferential hydratation (27). Although in another context, stabilization by ionic strength was also found for ADPGlc PPase from barley endosperm, since it was reported that ammonium sulfate fractionation stabilizes this enzyme to purification by a subsequent heat treatment procedure (28).

On the other hand, the protective effect of PEG on thermal inactivation of ADPGlc PPase may be attributed to the existence of a more hydrophobic environment for the protein in its presence. Previous results show that PEG exerts changes in the kinetic and regulatory allosteric properties of the cyanobacterial enzyme (29, 30). Thus, PEG elicits molecular crowding conditions, under which the response of ADPGlc PPase toward 3PGA performs with ultrasensitivity (30). It is concluded that the conformational change induced by PEG on the enzyme structure renders a protein with higher thermal stability.

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