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(Article begins on next page)

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Kinetic studies on rat liver low M_r phosphotyrosine protein phosphatases. The activation mechanism of the isoenzyme AcP2 by cGMP.

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Abstract

The reaction mechanisms of p-nitrophenyl phosphate hydrolysis catalyzed by two rat liver isoenzymes of the low M_r phosphotyrosine protein phosphatase (AcP1 and AcP2) were compared. Furthermore, the effect of some heterocyclic compounds on their activities were tested. Cyclic GMP and guanosine causes a particularly high activation of the isoenzyme AcP2, whereas its effect on AcP1 is very poor. A study on the mechanism of cyclic GMP activation was carried out. The results suggest that cyclic GMP activates the AcP2 isoenzyme by increasing the rate of the step that leads to the hydrolysis of the covalent enzyme-substrate phosphorylated complex formed during the catalytic process. The physiological significance of cyclic GMP activation of only one of the two isoenzymes (AcP2) remains uncertain.

Keywords: Phosphotyrosine protein phosphatase; Cyclic GMP; Isoenzymes

1. Introduction

Protein tyrosine phosphorylation plays a crucial role in the regulation of cell proliferation. Several phosphotyrosine protein kinases are involved in growth control and in cell transformation. The cellular phosphotyrosine level is regulated by the relative activities of opposing phosphotyrosine protein kinases and phosphotyrosine protein phosphatases (PTPases). PTPases are a family of enzymes that have no structural homology with the Ser/Thr-protein phosphatases. They are divided into two subfamilies [1,2]. The first one includes the receptor-like enzymes, whereas the other includes the non-receptor-like enzymes. The extracellular domains of the receptor-like PTPases LAR, DLAR, DPTP are homologous to cell adhesion molecules [3] and it was suggested that the interaction of these

receptor-like PTPases with the neighboring cells may serve as signal to activate their PTPase cytosolic domains, producing a counteraction against the phosphotyrosine protein kinases. Streuli [4] suggested that this mechanism is involved in the contact inhibition of cell growth. Nothing is known about the regulation of non-receptor-like PTPases.

This paper deals with the activation of a particular PTPase (E.C. 3.1.3.48), which was previously studied as an acid phosphatase (E.C. 3.1.3.2) by some purine compounds, particularly cGMP. This enzyme is localized in the cytosol and has a M_r of 18 000. Two isoenzymes that differ in residues 40–73 of the sequences have been isolated from both human erythrocytes and rat liver [5,6]. They probably originated from a single gene through an alternative splicing mechanism. We and other authors have recently demonstrated that this enzyme possesses a specific PTPase activity versus Tyr-phosphorylated protein and peptide substrates [7–11], and no activity on Ser-phosphorylated casein [10]. The enzyme catalyzes the hydrolysis of substrates by forming a thiol-phosphate covalent intermediate [12]. The hydrolysis of this intermediate is the limiting step of the catalytic process [13]. The same reaction mechanism is common exclusively to that of the

PTPase family members [1,2,14,15], and different from that shared by the acid and alkaline phosphatases [16].
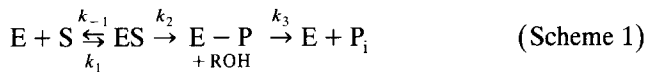
2. Materials and methods

Low M_r PTPase isoenzymes from rat liver (AcP1 and AcP2) were purified as previously described [6]. p-Nitrophenyl phosphate, phenyl phosphate, 2-naphthyl phosphate, L-phosphotyrosine, GMP, GDP, GTP, ATP, CMP, UMP, cGMP, cAMP, adenosine and guanosine were purchased from Sigma, St. Louis, MO. All other reagents were the purest commercially available.

2.1. Enzyme assay and transphosphorylation

The activity on p-nitrophenyl phosphate, phenyl phosphate, 2-naphthyl phosphate and L-phosphotyrosine was determined as follows: substrates were dissolved in 0.1 M sodium acetate buffer (pH 5.5) containing 1 mM EDTA. The reaction was started by adding small volumes of enzyme (final volume = 1 ml, 37°C) and stopped by adding 1 ml of 1 M NaOH. The phenols produced were measured spectrophotometrically by using the following extinction coefficients: $\epsilon_{400} = 18\,000$ $M^{-1}cm^{-1}$, p − nitrophenol [17]; $\epsilon_{287} = 2560$ $M^{-1}cm^{-1}$, phenol [13]; $\epsilon_{346} = 2780$ $M^{-1}cm^{-1}$, 2 − naphthol [13]; $\epsilon_{293.5} = 2330$ $M^{-1}cm^{-1}$, phosphotyrosine [18], all in alkaline solution. The extent of transphosphorylation from p-nitrophenyl phosphate to glycerol or methanol were estimated by measuring, in the same reaction mixture, the release of either p-nitrophenol or the release of P_i using the method of Baginski et al. [19].

2.2. Kinetic model for enzyme activation by cGMP

Zhang and Van Etten [13] proposed the following minimal scheme for the p-nitrophenyl phosphate hydrolysis catalyzed by the low M_r PTPase from bovine heart:

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} ES \overset{k_2}{\to} \underset{+\,ROH}{E - P} \overset{k_3}{\to} E + P_i \qquad \text{(Scheme 1)}$$

where E-P is a phosphorylated enzyme covalent complex. Taken into account that $k_2 \gg k_3$ and that the hydrolysis of the E-P covalent complex is the limiting step of the process [13], the kinetics of this type of process are formally described by the usual Michaelis-Menten two-steps scheme (Berezin et al. [20], Dissing et al. [21]):

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \overset{k_{cat}}{\to} E + P_i + ROH \qquad \text{(Scheme 2)}$$

where $K_s = k_{-1}/k_1$ is the true equilibrium dissociation constant of the enzyme-substrate complex (ES) and k_{cat} is the kinetic constant that leads to generation of products. Thus we applied this simple scheme to analyze the activa-

tion mechanism of rat liver AcP2 by cGMP. The following general scheme for non-essential activation (Segel [22]) was used (Scheme 3):
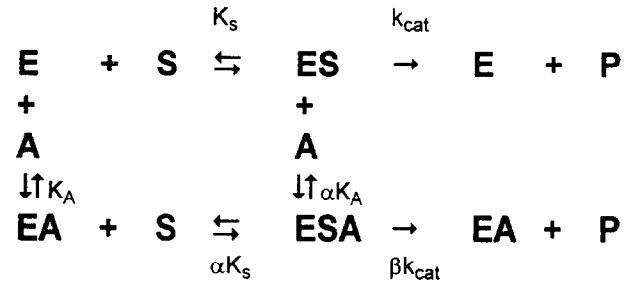
Scheme 3

where S is the substrate, A is the activator cGMP, P represents the products, K_A is the dissociation constant of the enzyme-cGMP complex (EA) into free enzyme (E) and cGMP, αK_S is the dissociation constant of the enzyme-activator-substrate complex (ESA) into EA and S, and αK_A is the dissociation constant for the ESA complex into ES and A, k_{cat} is the rate constant for the breakdown of ES into products, βk_{cat} is the rate constant for the breakdown of ESA into products.

The conservation equation for the enzyme is:

$$[E_t] = [E] + [ES] + [EA] + [ESA] \qquad (1)$$

The initial rate equation is:

$$v = k_{cat}[ES] + \beta k_{cat}[ESA] \qquad (2)$$

Dividing both sides by [Et]:

$$\frac{v}{[E_t]} = \frac{k_{cat}[ES] + \beta k_{cat}[ESA]}{[E] + [ES] + [EA] + [ESA]} \qquad (3)$$

From steady-state assumption for [ES]:

$$[ES] = \frac{[E][S]}{K_m} \; ; [EA] = \frac{[E][A]}{K_A} \; ; [ESA] = \frac{[E][S][A]}{\alpha K_A K_m}$$

where $(k_m = (k_{-1} + k_{cat})/k_1)$. Rearranging Eq. (3) and substituting for [ES], [EA], and [ESA]:

$$\frac{v}{k_{cat}[E_t]} = \frac{\dfrac{[E][S]}{K_m} + \dfrac{\beta[E][S][A]}{\alpha K_A K_m}}{[E] + \dfrac{[E][S]}{K_m} + \dfrac{[E][A]}{K_A} + \dfrac{[E][S][A]}{\alpha K_A K_m}} \qquad (4)$$

canceling E and substituting V_{max} for $k_{cat}[E_t]$:

$$\frac{v}{V_{max}} = \frac{\dfrac{[S]}{K_m} + \dfrac{\beta[S][A]}{\alpha K_A K_m}}{1 + \dfrac{[S]}{K_m} + \dfrac{[A]}{K_A} + \dfrac{[S][A]}{\alpha K_A K_m}} \qquad (5)$$

then, in Michalis-Menten form, the velocity equation is:

$$\frac{v}{V_{max}} = \frac{[S]}{K_m \dfrac{\left(1 + \dfrac{[A]}{K_A}\right)}{\left(1 + \dfrac{\beta[A]}{\alpha K_A}\right)} + [S] \dfrac{\left(1 + \dfrac{[A]}{\alpha K_A}\right)}{\left(1 + \dfrac{\beta[A]}{\alpha K_A}\right)}} \quad (6)$$

the reciprocal form of Eqn. (6) is:

$$\frac{1}{v} = \frac{K_m\left(1 + \dfrac{[A]}{K_A}\right)}{V_{max}\left(1 + \dfrac{\beta[A]}{\alpha K_A}\right)} \frac{1}{[S]} + \frac{1}{V_{max}} \frac{\left(1 + \dfrac{[A]}{\alpha K_A}\right)}{\left(1 + \dfrac{\beta[A]}{\alpha K_A}\right)} \quad (7)$$

If a plot of $1/v$ versus $1/[S]$ at different cGMP concentrations is made, a set of straight lines is obtained (see Fig. 2 below). The $1/v$ axis and $1/[S]$ axis intercepts of the limiting plot (at a saturating concentration of activator) give $1/\beta V_{max}$ and $-1/K_m$ [22]. In the absence of the activator Eq. (7) becomes the well − known Lineweaver-Burk equation [23]. Clearly, β represents the maximal activation at an infinitely high concentration of cGMP.

2.3. Determination of K_s, k_2 and k_3 relative to AcP1 and AcP2.

The determination of the true enzyme − substrate equilibrium dissociation constant $(k_s = k_{-1}/k_1)$ was achieved using a method previously described by Cirri et al. [24] and cited herein. This method takes advantage of the transphosphorylation properties of the enzyme from the substrate and an acceptor molecule that competes with water in the attack at the EP covalent complex (glycerol in our case).

3. Results and discussion

3.1. Kinetic properties of AcP1 and AcP2

The main kinetic properties of both rat liver AcP1 and AcP2 isoenzymes relative to four substrates have been determined (Table 1). It can be seen that AcP1 shows K_m values lower than those of AcP2 with respect to all tested substrates. Furthermore AcP1 has very similar kcat values for the four substrates. On the contrary AcP2 shows similar kcat values for p-nitrophenyl phosphate, phenyl phosphate and 2-naphthyl phosphate, but quite a lower value for L-phosphotyrosine. Zhang and Van Etten [13,25] showed that the bovine heart low M_r PTPase, which is strictly related to AcP2 [6], has quite constant kcat values for structurally differing aryl phosphates, including L-phosphotyrosine, and they concluded that the hydrolysis of all tested aryl phosphates proceeds through the same phosphoenzyme covalent intermediate, the hydrolysis of this intermediate being the limiting step of the catalytic process. The evidence relative to L-phosphotyrosine was contradicted by our findings in experiments of phosphotransfer from L-phosphotyrosine to glycerol [24] catalyzed by

Table 1

Kinetic parameters of rat liver AcP1 and AcP2 isoenzymes. A scheme of the structural differences between AcP1 and AcP2 is shown in the bottom.

		AcP1	AcP2
p-Nitrophenyl phosphate			
	k_{cat} (s^{-1})	15.0	29.8
	K_m (mM)	0.05	0.18
	K_s (mM)	0.46	5.40
	k_2 (s^{-1})	138	894
	k_3 (s^{-1})	16.8	30.8
L-Phosphotyrosine			
	k_{cat} (s^{-1})	14.6	12.1
	K_m (mM)	0.55	6.10
Phenyl phosphate			
	k_{cat} (s^{-1})	14.8	24.9
	K_m (mM)	0.49	1.46
2-Naphthyl phosphate			
	k_{cat} (s^{-1})	13.8	26.5
	K_m (mM)	0.06	0.46

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                         20                    40
AcP1 Ac-AEVGSKSVLFVCLGNICRSPIAEAVFRKLVTDENVSDNWRIDSAATSTYEVGNPPDYR
AcP2 ------------------------------------------A---S-V-DWN--R---P-
        60                    80                   100
     GQNCMKKHGIHMQHIARQITREDFATFDYILCMDESNLRDLNRKSNQVKNCRAKIELLGSY
     AV--LRN---STA-K---------------------------------------------
     120                   140
     DPQKQLIIEDPYYGNDSDFEVVYQQCLRCCKAFLEKTH-OH
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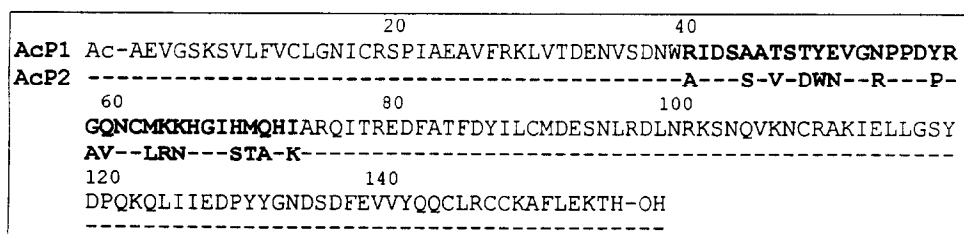
Table 2

Effect of some compounds on the activities of the rat liver AcP1 and AcP2 isoenzymes

Addition to the assay mixture	Relative activity	
	AcP1	AcP2
[a] None	100 ± 2	100 ± 2
[a] Guanosine	99 ± 2	240 ± 6
[a] GMP	107 ± 3	128 ± 3
[a] GDP	100 ± 3	120 ± 4
[a] GTP	100 ± 2	114 ± 2
[a] cGMP	105 ± 3	340 ± 8
[a] cGMP + 0.05 M NaCl	n.d.	340 ± 6
[a] cGMP + 0.1 M NaCl	n.d.	330 ± 7
[b] cGMP (pH 6.0)	n.d.	320 ± 5
[b] cGMP (pH 6.5)	n.d.	295 ± 6
[b] cGMP (pH 7.0)	n.d.	280 ± 4
[a] Adenosine	98 ± 1	100 ± 2
[a] cAMP	98 ± 1	113 ± 3
[a] ATP	$99 + 2$	$100 + 2$
[a] CMP	95 ± 2	96 ± 2
[a] UMP	100 ± 1	99 ± 2

[a] Assays performed with 4 mM p-nitrophenyl phosphate as substrate at 37°C and pH 5.5. [b] The assays in the pH range 6–7 were performed at higher p-nitrophenyl phosphate concentrations in order to saturate the enzyme, since the Km for this substrate increases with increasing pH. The final concentration of each added compound was 0.8 mM; n.d., not determined.

the bovine liver low M_r PTPase. In fact we found evidence that in the L-phosphotyrosine hydrolysis mechanism, k_2 (see Scheme 1) is partly rate-determining [24], even if the k_{cat} value is not significantly different from those of the other arylphosphates. L-phosphotyrosine is different from other tested aryl phosphates since it has additional carboxyl and amino groups that are not present in the other substrates tested by Zhang and Van Etten [25]. We suggested that these groups cause a lowering of the rate of enzyme phosphorylation [24]. Clearly, these groups are not present in the Tyr-phosphorylated proteins, since they are involved in the protein peptide backbone formation. The p-nitrophenyl phosphate hydrolysis mechanism, catalyzed by both AcP1 and AcP2, was studied. Using the method described in 'Materials and Methods', we were able to determine K_s (the true enzyme-substrate dissociation constant) values relative to both AcP1 and AcP2. In both cases K_s is higher than K_m (Table 1); this indicates that the mechanism of p-nitrophenyl phosphate hydrolysis catalyzed by both isoenzymes proceeds through an intermediate [26]. We also determined the kinetic constants k_2 and k_3 (see Scheme 1). Table 1 shows the resulting values. It can be seen that both AcP1 and AcP2 have k_2 values much higher than k_3 values, suggesting that, in both cases, the hydrolysis of E-P is the limiting step of the process.

3.2. The effect of heterocyclic compounds on AcP1 and AcP2

Several authors [27–33] have reported that certain heterocyclic compounds modulate the activity of low M_r PT-

Pase (previously known as low M_r acid phosphatase). Table 2 reports the effect of some heterocyclic compounds on the activity of both rat AcP1 and AcP2 isoenzymes. It can be seen that, at a final concentration of 0.8 mM, guanosine and cGMP strongly activated the AcP2 isoenzyme (2.4-fold and 3.4-fold, respectively), whereas the AcP1 isoenzyme was only slightly activated by cGMP. Guanosine has no effect on this latter form. The activation of AcP2 by cGMP was observed also at different pH values in the range 5.5–7.0 and is slightly modified by increasing ionic strength (Table 1). Furthermore, the noncyclic nucleotides GMP, GDP and GTP activate AcP2 1.28-fold, 1.20-fold and 1.14-fold, respectively. cAMP causes the activation of AcP2 (only 1.13-fold), while the other tested purine nucleotide compounds (adenosine and ATP) have little or no effect on the activity of both enzymes. The effect of the pyrimidine nucleotides CMP and UMP on both AcP1 and AcP2 activities was negligible. The particularly high AcP2 activation elicited by cGMP suggested to us to characterize better the activation mechanism and to discuss its possible physiological implications.

3.3. Activation mechanism of AcP2 by cGMP

Fig. 1 shows the activation of AcP2 at increasing concentrations of cGMP. It can be seen that the experimental points fit well with a hyperbolic curve. The maximal activation at infinitely high cGMP concentrations (asymptote of the curve) can be calculated. In the case of p-nitrophenyl phosphate used as substrate, we found a value of 6.1 that is the β-coefficient in Scheme 3.
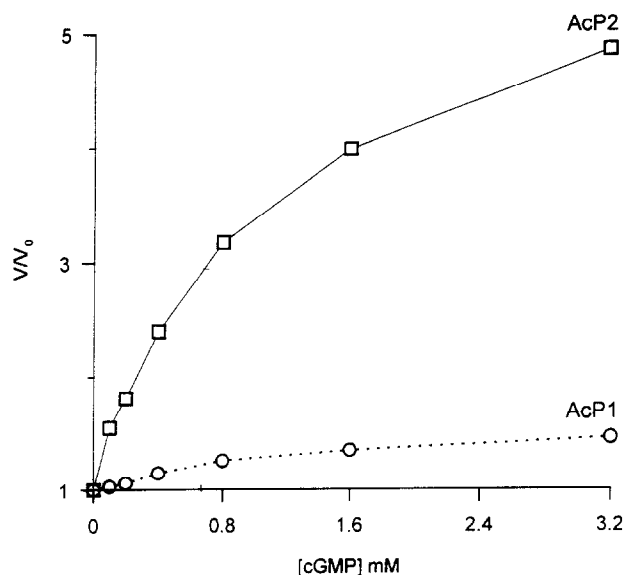

Fig. 1. Effect of cGMP concentration on the activity of AcP1 and AcP2 PTPase isoenzymes. V_0 is the relative V_{max} (taken as unit) in the absence of cGMP; V is the relative V_{max} in the presence of cGMP at the indicated concentrations.
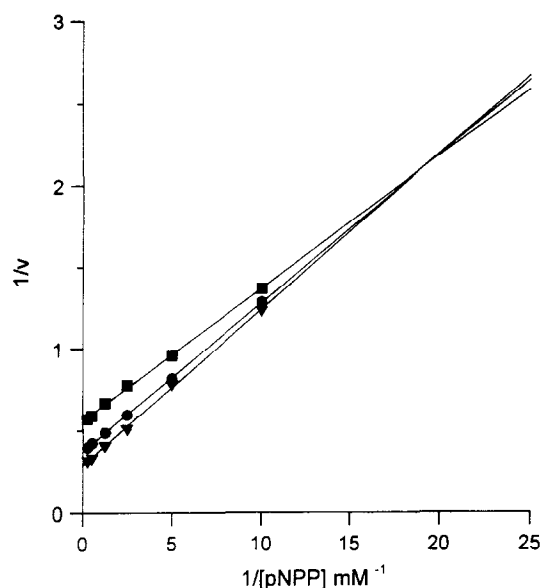
Fig. 2. Double reciprocal plot for the hydrolysis of p-nitrophenyl phosphate catalyzed by AcP2 in the presence of increasing concentrations of cGMP. The initial rates of p-nitrophenyl phosphate hydrolysis were measured at pH 5.5 and 37°C at the indicated concentrations either in the absence (■) or presence of (●), 0.1 mM cGMP, and (▼), 0.2 mM cGMP.

By plotting the reciprocal of the initial rates of p-nitrophenyl phosphate hydrolysis at varying concentrations of p-nitrophenyl phosphate and cGMP versus the reciprocal of substrate concentration (Eqn. 7) a series of straight lines were obtained (Fig. 2). These lines intersect at a point in the upper right quadrant. The intersection point has the following coordinates :

$$\text{ordinate} = \frac{1}{V_{max}} \left(\frac{\alpha - 1}{\alpha - \beta} \right) \text{ ; abscissa} = -\frac{(1 - \beta)}{K_m(\alpha - \beta)}$$

from which α = 7.95 (for p-nitrophenyl phosphate) can be calculated. We observe that both α and β are > 1 and that $\alpha > \beta$. This activation behavior can be classified as mixed-type activation as suggested by Segel [22].

Fig. 1 also shows that the effect of cGMP on AcP1 is negligible with respect to that observed for AcP2. The former enzyme does not form any complex between the free enzyme and cGMP as demonstrated by the spectrum showed in Fig. 3, which contains no differential bands. On the contrary, AcP2 gave a differential band near 220 nm (Fig. 3), confirming the formation of a complex between the free AcP2 isoenzyme and cGMP.

The mechanism of glycerol activation of the low M_r PTPase from bovine liver has been previously studied [24]. We found that the activation is caused by the interaction of glycerol with the E-P covalent complex. Glycerol directly attacks the E-P covalent complex (see Scheme 3) leading to phosphoglycerol(s) formation without affecting the P_i production rate, since the k_2 kinetic constant is much higher than the k_3 constant, leading to the accumulation of

the E-P covalent complex [24]. This is not what we have found with cGMP. Table 3 reports the results relative to the p-nitrophenol/P_i ratio produced by the enzyme action in the presence of glycerol or cGMP. It can be seen that there is no transphosphorylation from p-nitrophenyl phosphate to cGMP, since the p-nitrophenol/P_i ratio remains near the unit both in the absence and presence of cGMP. Clearly glycerol increases the enzyme activity through transphosphorylation as was previously found [24] .

Table 3 also reports the results of enzyme activation in the presence of both cGMP and glycerol. By increasing the cGMP concentration at a fixed concentration of glycerol (1 M), a decrease of tranphosphorylation from the substrate to glycerol was observed. This suggests that cGMP and glycerol interact with the enzyme at the same site. Alternatively the binding of cGMP with the free enzyme or with one of the intermediate forms hindered glycerol but not water access to the active site. We tested this latter hypothesis using methanol (a smaller molecule than glycerol) as acceptor. It can be seen that similar results were obtained (Table 3). These last results, although they do not exclude the possibility that both glycerol and methanol hindered the water attack at the E-P covalent complex, they do agree with the hypothesis that cGMP enhances the rate of the E-P hydrolysis by directly acting on the limiting step of the catalytic process (the water attack of the covalent
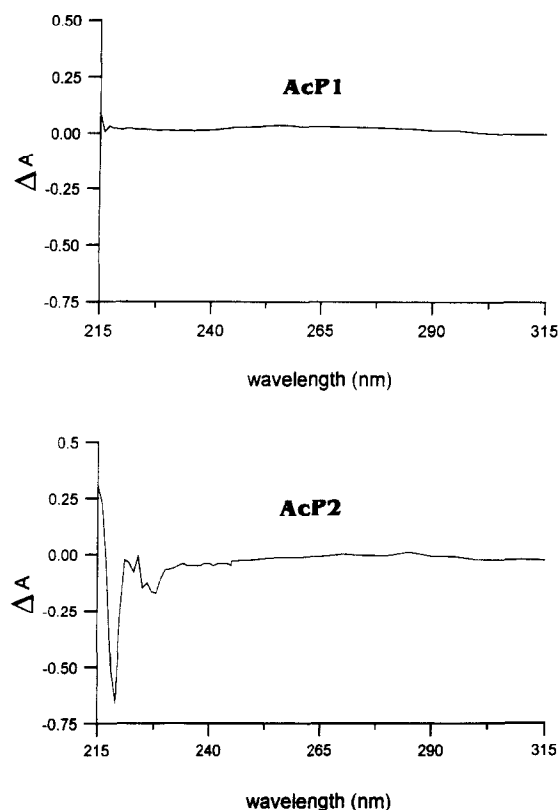

Fig. 3. Difference spectra of AcP1 and AcP2 PTPase isoenzymes (17 μM) in the presence of 0.1 mM cGMP. Spectra were recorded using a Beckman Model DU 7500 spectrophotometer.

Table 3

Rat liver AcP2 isoenzyme: effect of cGMP on the transphosphorylation to glycerol and methanol.

Addition to the assay mixture	p-nitrophenol/Pi	v/v_0
None	1.01 ± 0.02	1.00 ± 0.02
0.08 mM cGMP	0.97 ± 0.02	1.50 ± 0.03
0.10 mM cGMP	0.99 ± 0.01	1.74 ± 0.03
0.40 mM cGMP	0.96 ± 0.02	3.10 ± 0.06
0.50 mM cGMP	1.03 ± 0.03	3.51 ± 0.0
60.80 mM cGMP	1.05 ± 0.02	4.32 ± 0.05
1.00 mM cGMP	1.02 ± 0.02	5.43 ± 0.06
1.0 M Glycerol	3.06 ± 0.08	3.07 ± 0.02
1.0 M Glycerol + 0.10 mM cGMP	2.39 ± 0.04	3.17 ± 0.04
1.0 M Glycerol + 0.50 mM cGMP	1.98 ± 0.04	4.73 ± 0.05
1.0 M Glycerol + 1.00 mM cGMP	1.65 ± 0.03	5.81 ± 0.05
1.0 M Methanol	1.91 ± 0.04	1.90 ± 0.02
1.0 M Methanol + 0.08 mM cGMP	1.55 ± 0.02	2.10 ± 0.03
1.0 M Methanol + 0.40 mM cGMP	$1.32 + 0.03$	3.56 ± 0.03
1.0 M Methanol + 0.80 mM cGMP	1.18 ± 0.02	4.46 ± 0.04

All assays were performed using 8 mM p-nitrophenyl phosphate as substrate at 37°C and pH 5.5. v_0 is the initial velocity measured in the absence of activators; v is the initial velocity measured in the presence of activators.

complex thiol-phosphate bond). This is clearly indicated by the decreasing p-nitrophenol/P_i ratio at increasing cGMP concentrations in the presence of 1 M glycerol while the v/v_0 ratio increases. The lower acceptor capability of glycerol in the presence of cGMP might be due to the lowering of the steady-state E-P concentration caused by the increased k_3.

The determination of the β-coefficients relative to other substrates (phenyl phosphate and 2-naphthyl phosphate) gave results that agree well with the hypothesis that cGMP increases the rate of dephosphorylation of the phosphorylated enzyme-complex. In fact, β = 6.2 for phenyl phosphate and β = 5.9 for 2-naphthyl phosphate were found. These data are very close to the value of β = 6.1 for p-nitrophenyl phosphate. Since there are structural differences among p-nitrophenyl phosphate, phenyl phosphate and 2-naphthyl phosphate, the similarity of the β values indicates a common step (the dephosphorylation of the covalent complex) as the target of the cGMP action.

In the cell cGMP is produced by both membrane-bound and soluble GMP cyclases. The former is strongly stimulated by some substances such as the atrial natriuretic factor or the *E. coli* heat-stable enterotoxins, whereas the latter (a heme-containing enzyme) is strongly stimulated by nitric oxide [34]. Table 3 shows that 80 μM cGMP concentration causes 50%-activation of the AcP2 isoenzyme in vitro. Although the mean cGMP concentration in the cell is lower than 80 μM, the local concentration in the sites of cGMP production may increase during activation of the cyclases, reaching levels able to stimulate the PT-Pase activity in vivo.

Acknowledgements

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