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EVIDENCE FOR A ROLE OF SULFHYDRYL GROUPS IN CATALYTIC ACTIVITY AND SUBUNIT INTERACTION OF THE CYCLIC GMP-DEPENDENT PROTEIN KINASE FROM SILKWORM

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Summary

Guanosine 3′:5′-monophosphate-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) has been isolated from silkworm pupal fat body (*Bombyx mori*) which is devoid of any adenosine 3′:5′-monophosphate-dependent protein kinase. The enzyme displayed catalytic properties which were roughly similar to those described for adenosine 3′:5′-monophosphate-dependent protein kinase. This similarity has been found in substrate specificity, optimal Mg^{2+} dependency, polyamines effects and the lack of dependency upon sulfhydryl compound for activation by cyclic GMP. Treatment of the enzyme with sulfhydryl reagents, *N*-ethylmaleimide or *p*-chloromercuribenzoic acid, inhibited the catalytic activity as well as the dissociation of the binding and catalytic activities as shown by means of sucrose-density gradient ultracentrifugation. In the presence of cyclic GMP or histone, the disulfide-linked structure did not dissociate into separate subunits nor did it migrate as the holoenzyme but sedimented as an intermediate form carrying both binding and catalytic activities.

Introduction

Since the discovery of a cyclic GMP[1]-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) in lobster tail muscle by Kuo and Greengard [1], cyclic GMP-dependent enzymes from several invertebrate and vertebrate tissues have been purified [1—7]. Most of the properties of those cyclic GMP-dependent protein kinases which have been characterized, displayed a great heterogeneity.

Abbreviations: MES, 2(*N*-morpholino)ethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl-ether)-*N*,*N*′tetraacetic acid.

For instance, the cyclic GMP-dependent enzyme purified from bovine cerebellum [6] and pig and bovine lung [8—10] differed from the enzyme preparation purified from foetal guinea-pig lung [7], foetal calf heart [11], bovine oarta [12] and lobster tail muscle [13] in that these preparations did not require the stimulatory modulator for the cyclic GMP stimulation. Also, different reports have emphasized some discrepancies in the Mg^{2+} concentration required for optimal enzyme activity ranging from 5 to 100 mM [3,6,8,11,12]. However, the major subject of controversy concerned the mode of activation of the enzyme: a number of studies indicated that cyclic GMP promotes dissociation of the cyclic GMP-dependent protein kinase [4,11,12,14,15,20] suggesting the cyclic nucleotide-induced activation occurred according to the scheme demonstrated for cyclic AMP-dependent protein kinases [16—19], whereas several other studies have shown that cyclic GMP binding and consequent activation of the enzyme did not involve any subunit dissociation [9,10,21,22]. Furthermore, Takai et al. [21] found that, unlike the other cyclic GMP-dependent protein kinases, the enzyme from *Bombyx mori* had an absolute requirement for an exogenous sulfhydryl compound for its activation by cyclic GMP. Thus, the present study was undertaken in order to determine the possible role of thiol groups in the enzyme activity employing cyclic GMP-dependent protein kinase from silkworm (*B. mori*) pupal fat body. The data reported herein provide evidence for a role of sulfhydryl groups in the activation and dissociation processes and confirm the close similarities between cyclic AMP- and cyclic GMP-dependent protein kinase properties *.

Materials and Methods

Cyclic GMP-dependent protein kinase was prepared from silkworm pupae (*B. mori*) which had started their pupal development at room temperature. Silkworms were used between the first and the third day after larval-pupal ecdysis, when the fat body was still abundant and devoid of cyclic AMP-dependent protein kinase [2,24]. The abundant spongy tissue which fills most of the space between the intestine and body wall was quickly removed from the pupae, rinced once with ice-cold lepidopterian Locke's solution and homogenized 1 min with 3 vols. ice-cold Buffer A (20 mM potassium phosphate (pH 7.0)/6 mM 2-mercaptoethanol/2 mM EDTA/5 mM phenylmethyl sulphonyl fluoride **).

All operations were performed at 0—4°C. The homogenate was centrifuged for 30 min at 27000 $\times g$ and the cyclic GMP-dependent protein kinase was prepared from cytosol using the acetic acid and $(NH_4)_2SO_4$ precipitation steps described by Kuo et al. [1]. The final precipitate was then dissolved in Buffer A and dialysed overnight against 20 vols. of the same buffer. The insoluble material was removed by centrifugation and the supernatant solution adjusted to a final concentration of 10 mg protein/ml with Buffer A. The catalytic and

* Part of these results was presented at the Third International Conference on Cyclic Nucleotides, New Orleans, Louisiana, U.S.A., July 1977.

** The final concentration in Buffer A of the phenylmethylsulphonylfluoride solvent, dimethylsulfoxide was 0.5%.

cyclic GMP-binding properties of the enzyme preparation were stable for one week when stored under these conditions on ice.

Protein kinase activity was assayed by the following procedure: the reaction was conducted in 80 μl containing 25 mM MES buffer (pH 7.2), 0.25 mM EDTA, 5 mM $MgCl_2$, 2.5 mM theophylline, 125 μM [γ-^{32}P]ATP (specific activity 20 cpm/pmol), 300 μg mixed histone (Sigma type IIA) and 0.1 μM cyclic GMP (where indicated). The reaction was initiated with the addition of 100 μg enzyme preparation and the tubes were incubated for 15 min at 30°C. The reaction was terminated by spotting 50 μl assay mixture onto squares of Whatman P 81 phosphocellulose paper and washing as described by Witt et al. [2]. All measurements of the adsorbed radioactivity were made in an Intertechnique scintillation spectrometer measuring Cerenkov radiation. The results were expressed as pmol phosphate incorporated into histone/min per mg protein. Protein was determined by the method of Lowry et al. [26] with bovine serum albumin as a standard.

Cyclic GMP binding assay was performed on aliquots of gradient fractions in a 0.1 ml total volume containing 50 mM sodium acetate (pH 4.0), 20 nM cyclic [^{3}H]GMP and 10 μg bovine gamma globulin as carrier. All subsequent steps were similar to those previously described [27].

Protein kinase modulator was prepared from rat liver through the trichloracetic acid step as described elsewhere [28].

[γ-^{32}P]ATP (17 Ci/mmol), cyclic [^{3}H]GMP (31 Ci/mmol) and Scintix were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Polyamines, protein substrates and other chemicals were purchased from Sigma Chemical Company, St. Louis, Mo, U.S.A. The silkworm pupae were a generous gift from Pr. J. Daillie (Lyon).

Results

Characterization of enzyme

The cyclic GMP-dependent protein kinase from silkworm pupae displayed a high affinity and specificity for cyclic GMP. As shown in Fig. 1, the apparent K_a value for this cyclic nucleotide was 1.4 nM, whereas the cyclic AMP concentration needed for half-maximal stimulation was 500-fold higher (0.8 μM).

The silkworm protein kinase required divalent ions for activity. Fig. 2 shows that the enzyme was maximally active at about 5 mM Mg^{2+} with mixed histone as protein substrate. Co^{2+} was active with an optimal concentration of 2 mM and found to be less effective than 5 mM Mg^{2+}. Other divalent ions, Mn^{2+} and Zn^{2+}, were also tested and were nearly ineffective. Even though it had no effect when present alone, Ca^{2+} antagonized the stimulatory effect of 5 mM Mg^{2+} in a dose-related manner (data not shown).

The relative ability of protamine and various histone subfractions at saturating concentrations to serve as phosphate acceptor for the enzyme activity decreased in the order: H2b > protamine > H1 > mixed histone > H3 > H2a. Casein, as well as phosvitin, was a poor substrate for the enzyme. However, the data given in Fig. 3 show that the casein phosphorylation was enhanced by polyamines under conditions which decreased mixed histone phosphorylation.

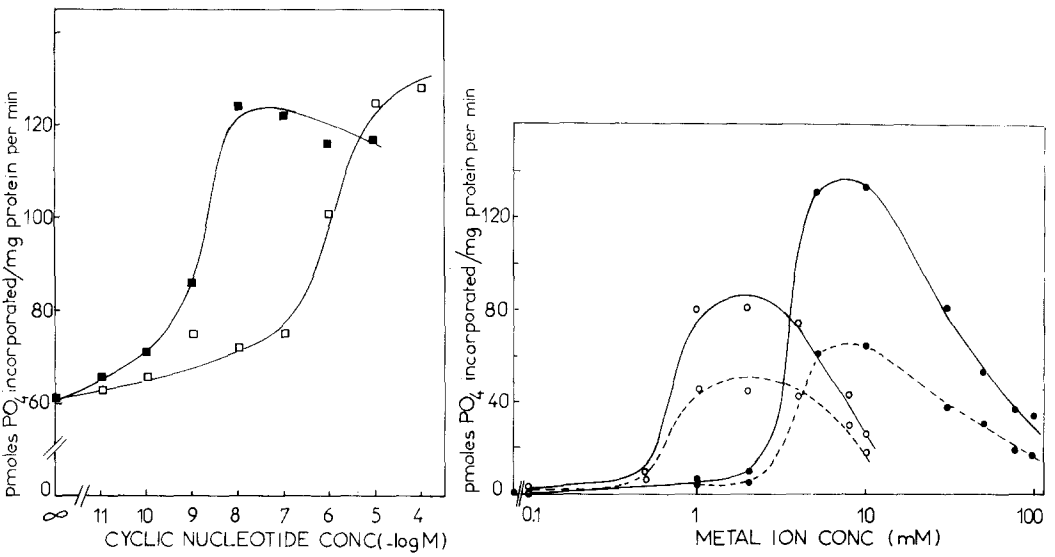


Fig. 1. Specificity of cyclic GMP-dependent protein kinase partially purified from silkworm pupae for cyclic GMP (■) and cyclic AMP (□). The assay conditions were as described in Materials and Methods.

Fig. 2. Relative effects of Mg^{2+} (●) and Co^{2+} (○) on silkworm pupae protein kinase activity assayed in the presence (———) or absence (- - - - -) of 0.1 μM cyclic GMP as described under Fig. 1 except for variations in the kind and concentration of divalent ions.

Activity and sedimentation behavior of the cyclic GMP-dependent protein kinase

Role of sulfhydryl groups. Reducing agents such as 2-mercaptoethanol were not needed either for the basal activity of the enzyme nor for the stimulation by cyclic GMP (table I), while the results given in Fig. 4 show that catalytic activity was strongly inhibited by reagents such as *p*-chloromercuribenzoate

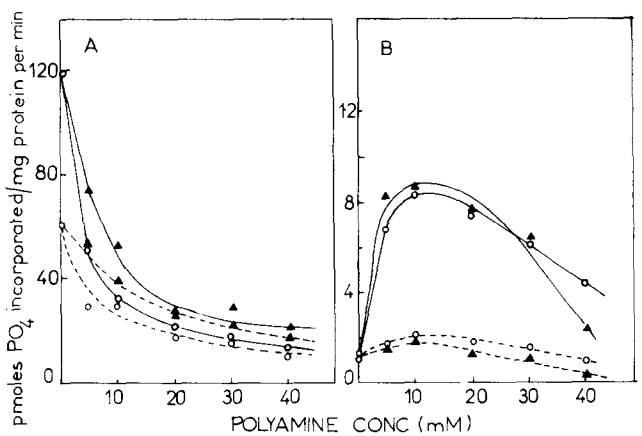


Fig. 3. Effect of polyamines on the cyclic GMP-dependent protein kinase. (A) Enzymatic activity was assayed in the presence (———) and absence (- - - - -) of 0.1 μM cyclic GMP under standard conditions except that spermine (○) or spermidine (▲) were added at increasing concentrations. (B) Same as (A) except that casein (600 μg) was used as protein substrate.

TABLE I

EFFECT OF 2-MERCAPTOETHANOL ON CYCLIC GMP-DEPENDENT PROTEIN KINASE FROM SILKWORM PUPAE

The enzyme was dialysed for 24 h against 20 vols. 20 mM potassium phosphate (pH 7.0) at 0°C in order to remove endogenous 2-mercaptoethanol. Then protein kinase activity was assayed under standard conditions except that 2-mercaptoethanol was added as indicated. When present, cyclic GMP was 0.1 μM.

2-Mercaptoethanol concentration (mM)	Protein kinase activity (pmol phosphate incorporated/mg protein per min)	
	— Cyclic GMP	+ Cyclic GMP
0	60.0	118
1	68.1	114
5	62.8	109
10	61.7	106
20	61.0	121
30	64.8	116

and *N*-ethylmaleimide which react with sulfhydryl groups. Under the conditions used, *p*-chloromercuribenzoate-induced inhibition was reversed by restoring a 6 mM 2-mercaptoethanol concentration whereas the inhibition by *N*-ethylmaleimide was irreversible.

In order to determine whether the sulfhydryl reagents affected the enzyme dissociation, the effect of *p*-chloromercuribenzoate on the sedimentation behavior of the enzyme was studied.

The cyclic GMP-dependent activity of the silkworm enzyme sedimented in a sucrose-density gradient as a holoenzyme at a position corresponding to 6.6 S with a minor shoulder of more slowly migrating components (Fig. 5A). It must be stressed that the cyclic GMP-stimulated activity of the unfraction-

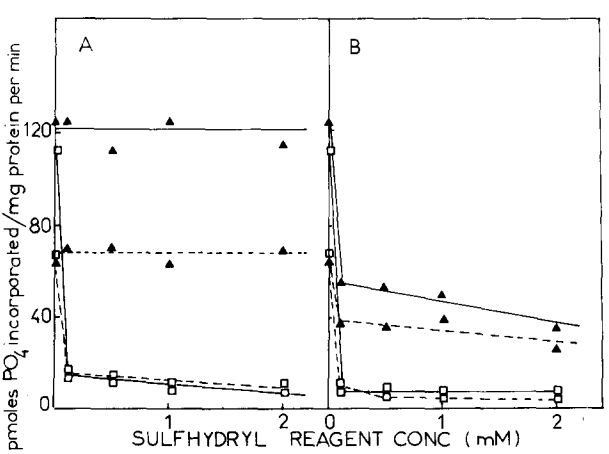
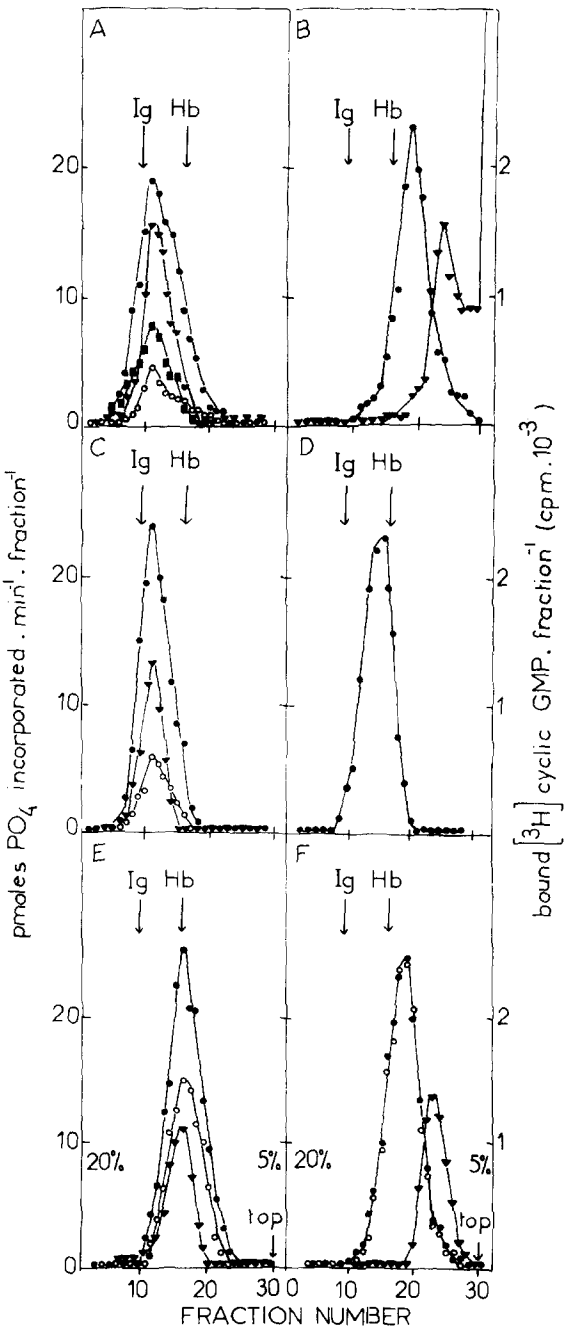


Fig. 4. Inhibition of catalytic activity by sulfhydryl group reagents. Enzyme was preincubated with various concentrations of *p*-chloromercuribenzoate (A) or *N*-ethylmaleimide (B) for 15 min at 0°C and the preincubation allowed to go on for another 15 min on ice in the presence (▲) or absence (□) of 6 mM 2-mercaptoethanol. Protein kinase assay was initiated by adding the reaction mixture described in Materials and Methods with (———) or without (- - - - -) 0.1 μM cyclic GMP.

ated preparation was much lower than the one obtained when the gradient fractions were used. This higher cyclic GMP dependency was presumably due to separation from inhibitory materials present in the impure preparation. No greater stimulation was achieved in the presence of the protein kinase modu-



lator when mixed histone was used as substrate. A 2-fold stimulation of activity was also obtained at the same position when assayed with 1 μM cyclic AMP, which is close to the K_a value for this cyclic nucleotide. The cyclic GMP binding activity coincided with the enzyme activity.

The enzyme preincubation with 1 μM cyclic GMP shifted the pattern of catalytic activity to a single symmetric peak exhibiting a sedimentation coefficient of 3.7 S (Fig. 5B). For the purpose of locating the cyclic GMP binding component, 1 μM cyclic [^{3}H]GMP was included in the preincubation mixture. Then radioactive cyclic GMP was associated with a peak sedimenting in a position of about 2.4 S. The cyclic GMP binding activity of the loaded sample was quantitatively recovered in the gradient fractions suggesting that the cyclic GMP binding subunit was stable under the sedimentation conditions. Protamine and mixed histone were also able to substitute for cyclic GMP in causing the enzyme dissociation (data not shown).

The sulfhydryl reagent-treated enzyme sedimented as a single molecular species at the holoenzyme position (Fig. 5C). Under these conditions the comigrating catalytic and cyclic GMP binding activities were assayed after regeneration with 2-mercaptoethanol. The *p*-chloromercuribenzoate-treated enzyme did not shift towards the 3.7 S and 2.4 S positions when 1 μM cyclic GMP (Fig. 5D) or mixed histone (Fig. 5E) were included throughout the gradient, but a cyclic GMP-dependent intermediate species was formed with a sedimentation coefficient of 4.6 S bearing both catalytic and cyclic GMP binding activities. In contrast, addition of 6 mM 2-mercaptoethanol to the disulfide-linked enzyme before the run restored the dissociation by mixed histone (Fig. 5F).

Discussion

Based upon several physical and catalytic properties, a homology between cyclic AMP- and cyclic GMP-dependent protein kinases has been proposed by

Fig. 5. Sucrose-density gradient centrifugation of the cyclic GMP-dependent enzyme from silkworm pupal fat body. (A) Control gradient. The holoenzyme (0.5 mg) was preincubated in Buffer A for 30 min on ice. Then the sample was layered over a 5—20% linear sucrose-density gradient (4.8 ml) containing 25 mM Tris · HCl buffer at pH 7.0, 2 mM EDTA and 6 mM 2-mercaptoethanol. Sedimentation was performed at 200000 × *g* for 15 h at 2°C. Fractions of 0.16 ml were collected and 40 μl were assayed for protein kinase activity (with (●———●) and without (○———○) 0.1 μM cyclic GMP, with 1 μM cyclic AMP (■———■), and for cyclic [^{3}H]GMP binding activity (▲———▲). Sedimentation coefficients were determined by the method of Martin and Ames [32] using human immunoglobulins (Ig, 7.2 S), bovine creatine kinase (5.2 S) and human hemoglobin (Hb, 4.6 S) as internal markers. (B) Same as (A) except that 1 μM cyclic GMP was included in buffer A and throughout the gradient. Fractions were assayed for protein kinase activity (●———●). A parallel experiment was performed with 5 μCi cyclic [^{3}H]GMP (final concentration 1 μM) in buffer A and 0.01 μM cyclic GMP throughout the gradient. Cyclic [^{3}H]GMP binding activity (▲———▲) was determined by counting aliquots of each fraction in the presence of a scintaillation mixture (Scintix). (C) Holoenzyme (50 μl) was preincubated for 30 min on ice with 10 μl of 30 mM *p*-chloromercuribenzoate (final concentration of 0.5 mM). Centrifigation was performed as in (A) except that the gradient was devoid of 2-mercaptoethanol. Protein kinase activity was assayed after adding 5 μl of 0.2 M 2-mercaptoethanol to each fraction. (D) Holoenzyme was preincubated as in (C) and then for 30 min further with 1 μM cyclic GMP in Buffer A without 2-mercaptoethanol. Centrifugation was performed as in (A) except that 1 μM cyclic GMP was included in the gradient without 2-mercaptoethanol. Protein kinase activity was assayed as described in (C). (E) Same as (D) except that mixed histone (1 mg/ml) were included in Buffer A and throughout the gradient in the absence of 2-mercaptoethanol. (F) The enzyme was preincubated with *p*-chloromercuribenzoate as in (C). Before the run the sample was brought to a 6 mM 2-mercaptoethanol concentration by adding 10 μl of 40 mM 2-mercaptoethanol and centrifuged with mixed histone (1 mg/ml) present throughout the gradient.

Lincoln and Corbin [29]. The most striking similarities between the two enzymes were found in substrate specificity. Both enzymes catalysed phosphorylation of histone, pyruvate kinase, glycogen synthase, phosphorylase kinase and hormone-sensitive lipase [29,30].

The properties of cyclic GMP-dependent protein kinase from silkmoth pupal fat body, which have been reported herein, provide evidence supporting the homology between the two kinds of enzymes. This analogy in substrate specificity has been confirmed for the following substrates: histone, protamine, casein and phosvitin. Additional similarities have also been emphasized, such as the Mg^{2+} concentration required for optimal activity, the effects of polyamines when either histone or casein were used as protein substrates, the lack of dependency upon sulfhydryl compounds for activity and the cyclic nucleotide or substrate-induced dissociation of the enzyme. The apparent K_a value of the cyclic GMP silkmoth pupal enzyme for the specific cyclic nucleotide was much lower than those generally obtained with cyclic AMP enzymes. However, the most significant difference found concerned the heat-stable modulator which did not affect the enzyme activity.

The enzyme treatment with sulfhydryl reagents such as *p*-chloromercuribenzoate or *N*-ethylmaleimide inhibited the catalytic activity as well as the cyclic GMP- or histone-induced dissociation of the binding and catalytic activities. Sulfhydryl reagents caused a similar inhibition of the catalytic activity from skeletal muscle cyclic AMP-dependent protein kinase [31]. In the presence of cyclic GMP or histone, the disulfide-linked structure did not dissociate into separate subunits nor did it migrate as the holoenzyme (6.6 S form), but sedimented as a protomer (4.6 S form) bearing both binding and catalytic activities. These data suggest that disulfide bridges are involved in the interaction of the binding and catalytic components in addition to the peptide bonds which have also been implicated in the same interaction [22—23].

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