STUDIES ON CYCLIC GMP-DEPENDENT PROTEIN KINASE PROPERTIES BY BLUE DEXTRAN-SEPHAROSE CHROMATOGRAPHY

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Summary: Cyclic GMP-dependent protein kinase prepared from calf lung was studied for its binding properties with blue dextran-Sepharose affinity column chromatography. Blue dextran competitively inhibited [^{3}H]cGMP binding to the enzyme. ATP + Mg^{++} did not prevent cGMP-kinase binding to blue dextran, nor did it facilitate the liberation of blue dextran-bound enzyme. Substrate proteins such as histone and protamine dissociated the native enzyme into subunits. Considering all these results, cGMP-kinase seemed to conform with the "dissociation model" proposed for cAMP-kinase but with peculiarities of binding to blue dextran.

Protein kinase activated by cyclic GMP* rather than by cyclic AMP has been partially purified from lobster muscle (1), silk worm (2), bovine cerebellum (3), and pig lung (4). Cyclic GMP-kinase differs from cAMP-kinase in many catalytic, kinetic, and physicochemical properties (2-4). However, the enzymatic activity of both kinases must require binding steps at 3 different sites in the molecule: the binding of cyclic nucleotide for activation, the binding of ATP and Mg^{++}, and the binding of protein substrate for the reaction. The mechanism of cAMP-kinase activation involves first, the binding of cAMP to a regulatory subunit of the native enzyme, and the subsequent dissociation of a catalytic subunit to stimulate the phosphorylation of the substrate protein by ATP and Mg^{++} (5-7). In the present studies, using blue dextran-Sepharose affinity chromatography, we have explored all these binding sites of cGMP-kinase, and we suggest a similar mechanism of activation.

* Abbreviations: Adenosine-3′,5′-monophosphate, cyclic AMP or cAMP; cyclic AMP-dependent protein kinase, cAMP-kinase; guanosine-3′,5′-monophosphate, cyclic GMP or cGMP; cyclic GMP-dependent protein kinase, cGMP-kinase; ethylenediamine-tetraacetic acid, EDTA; ethyleneglycol-*bis*-(β-aminoethyl ether)N,N′-tetraacetic acid, EGTA.

MATERIALS AND METHODS

Isotopes and chemicals: [^{3}H]cGMP (21 Ci/mmole) and [γ^{32}P]ATP (0.76 Ci/mmole) were purchased from Amersham/Searle Corp., Arlington Heights, Ill. Unlabeled nucleotides, histone (type II), protamine sulfate, and DEAE-cellulose (0.88 mEq/g, medium mesh) were purchased from Sigma Chemical Co., St. Louis, Mo. Sephadex G-200, blue dextran 2000, and Sepharose 4B were supplied by Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

Preparation of cGMP-kinase: Cyclic GMP-kinase was extracted from calf lung using the method described by Nakazawa and Sano (4). The apparent Km for cGMP was 16.7 nM; for cAMP, it was 400 nM, identical to the values given in the original report (4).

Preparation of blue dextran-Sepharose column: The covalently linked blue dextran and Sepharose complex was prepared according to the procedure devised by Ryan and Vestling (8). It was packed into a column of 0.9 (I.D.) x 3 cm. Unless specified otherwise, the eluent was 10 mM potassium phosphate buffer, pH 7.0, containing EDTA (2 mM), 2-mercaptoethanol (50 mM), and glycerol (10%). Affinity chromatography of cGMP-kinase was carried out at 4° C.

Assay for cGMP binding activity: The binding activity of cGMP-kinase was assayed by measuring the binding of [^{3}H]cGMP to the enzyme protein, which was separated on a Millipore filter (HAWP 02500) under the conditions described previously (9).

Assay for kinase activity: Enzymatic activity of cGMP-kinase was assayed for protein substrate phosphorylation. The reaction took place in 0.2 ml of potassium phosphate buffer (10 mM), pH 7.0, containing theophylline (0.5 µmole), NaF (0.02 µmole), magnesium acetate (10 µmoles), EGTA (0.6 µmole), [γ^{32}P]ATP (1 nmole), calf thymus histone (150 µg), and the enzyme protein (42 or 21 µg in 50 or 25 µl). Cyclic GMP (1 or 5 µM) was added when activation was tested. Incubation was carried out for 5 min at 30° C, and the reaction was terminated by precipitation of histone with 4 ml of trichloroacetic acid (5%)-tungstate (0.25%) solution. The precipitate was washed 3 times with the same solution (4 ml each time), as described by Kuo et al. (10). The final precipitate was dissolved in 0.1 ml of 1 N NaOH for determination of its radioactivity by liquid scintillation spectrometer.

RESULTS

Affinity chromatography of cGMP-kinase and effects of nucleotides and Mg^{++}: Since the chromophore of blue dextran is a nucleotide analog, cGMP-kinase has been found to be retained in the blue dextran-Sepharose column. As shown in Table 1 (cGMP binding activity alone), and Figure 1A (both binding and kinase activities), only a small fraction of the enzyme bound to the column could be eluted in phosphate buffer eluate. Most of the enzyme (90%) was eluted in 0.2 M KCl. Incorporation of nucleotides and/or Mg^{++} in the buffer eluent did not increase the cGMP binding activity in the

Table 1. A lack of nucleotide and Mg^{++} effect on elution profile: [^{3}H]cGMP binding activities (cpm/100 µl*) in eluates† from a blue dextran-Sepharose affinity column of cGMP-kinase

Additive in eluent 2	Eluate 1	Eluate 2	Eluate 3
-	229	245	2902
Mg^{++}	183	95	1973
ATP	199	151	1754
ATP + Mg^{++}	195	82	1802
ADP + Mg^{++}	233	85	2636
AMP + Mg^{++}	254	96	2736
GTP + Mg^{++}	209	91	1821

* Mean value in duplicate.

† Eluate 1 was 3 ml of buffer eluent, eluate 2 was 5 ml of buffer eluent containing Mg^{++} (10 mM) and/or nucleotide (2 mM), and eluate 3 was 5 ml of buffer eluent containing KCl (0.2 M). One ml (840 µg) of enzyme was applied on the column and eluted in the order indicated by the eluents.

eluate (Table 1, eluate 2). Prior incubation of cGMP-kinase with ATP and Mg^{++} (Figure 1D) did not prevent the binding of the enzyme protein to the column, since the bound enzyme could still be eluted almost completely in 0.2 M KCl containing ATP and Mg^{++}. These results clearly indicate that the binding between cGMP-kinase and blue dextran does not involve the ATP/Mg^{++} site of the enzyme molecule.

Blue dextran competition at the cGMP binding site: When aliquots of the same cGMP-kinase preparation were mixed with blue dextran at various concentrations (2.5 mg - 2.5 µg/ml), both cGMP binding and protein kinase activities of the enzyme were inhibited by blue dextran at a concentration of 2.5 mg/ml, but not at a concentration of 250 µg/ml or less (Table

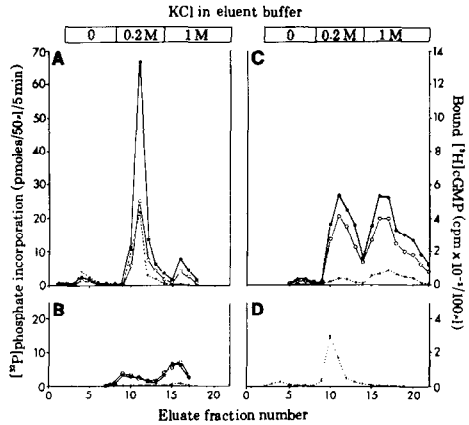

Figure 1. Blue dextran-Sepharose affinity column chromatography of cGMP-kinase (840 µg of protein) after incubation in 2 ml of phosphate buffer alone (A), containing 350 µg/ml of histone (B), 800 µg/ml of protamine (C), or 2 mM of ATP plus 10 mM of Mg^{++} (D) at 0° C for 15 min. The columns were preequilibrated with the above solutions and eluted with the same solutions containing the specified concentrations of KCl in each individual chromatograph. Each fraction of eluate (1 ml) was assayed for basal kinase activity (o——o), cGMP (1 µM)-activated kinase activity (●——●), and [^{3}H]cGMP binding activity (*---*).

2). The kinetics of [^{3}H]cGMP binding to the enzyme were analyzed in the presence of 1.25 mg and 625 µg/ml of blue dextran and compared to the control The double reciprocal plots illustrated in Figure 2 clearly demonstrate a competitive inhibitory effect of blue dextran at the cGMP binding site of the enzyme.

Attempts were made to elute the bound cGMP-kinase from the blue dextran-Sepharose affinity column by an eluent containing cGMP. We did not succeed with such experiments, however, because the cyclic nucleotide could not be liberated completely from its enzyme complex and thus would inevitably interfere with the [^{3}H]cGMP binding assay (11). Furthermore, the enzyme was rendered unstable once it was bound by the cyclic nucleotide.

Effects of substrate proteins on affinity chromatography: It has been found that the proton-rich substrate proteins, such as histone and protamine, were able to dissociate some cAMP-kinase preparations (12-14) and also to

Table 2. Effects of blue dextran on cGMP-kinase§

Final conc. of blue dextran (μg/ml)	[^{3}H]cGMP binding* (cpm/50 μl)	Protein kinase activity† (pmoles of [^{32}P] incorporated/25 μl/5min)	
		-cGMP	+cGMP (5 μM)
0 (control)	3573.0 ± 179.9	86.1	237.3
2500	148.6 ± 13.6	4.2	17.7
250	2779.5 ± 89.6	130.2	338.5
25	3862.9 ± 102.3	102.0	275.8
2.5	4074.9 ± 83.5	86.2	238.8

§ Enzyme concentration: 840 μg of protein per ml.

* Mean ± S.D. in triplicate.

† Mean value in duplicate.

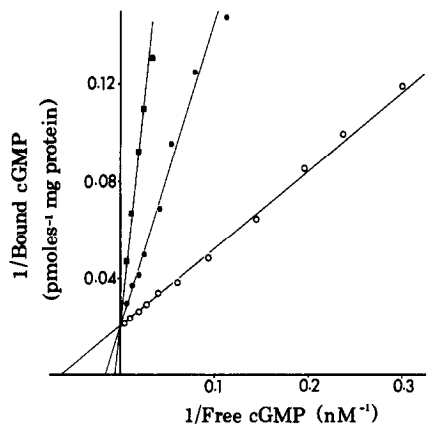

Figure 2. Reciprocal plots of the cGMP binding kinetics of cGMP-kinase (●) and the inhibitory effect of blue dextran at 625 μg/ml (o) and 1.25 mg/ml (■).

cause partial dissociation of lobster cGMP-kinase (11) into subunits. We therefore tested the effects of substrate-enzyme interaction by incubating cGMP-kinase with histone and protamine prior to affinity chromatography.

In the absence of substrate preincubation as control, [^{3}H]cGMP binding activity, and both the basal and cGMP-stimulated kinase activities were mainly present in the 0.2 M KCl eluate. The remaining small fractions were distributed in buffer and 1 M KCl eluates (Figure 1A). After preincubation with 350 µg/ml of histone, a different chromatogram (Figure 1B) was observed, in which kinase activity was absent in phosphate buffer eluate, reduced in 0.2 M KCl, but remained in 1 M KCl. It was no longer possible for the kinase activity to be stimulated by cGMP (1 µM), indicating that it might represent only the dissociated catalytic subunit. [^{3}H]cGMP binding activity was absent in the buffer and 0.2 M KCl fractions but detectable in the 1 M KCl fraction (Figure 1B). Preincubation with 800 µg/ml of protamine produced another distinctly different chromatogram (Figure 1C). With the exception of a little peak in the buffer eluate, almost equal fractions of kinase activity were present in 0.2 M and 1 M KCl eluates. The kinase activity was stimulated by 1 µM cGMP to only 30% above basal level. [^{3}H]cGMP binding activity was distributed in all 3 fractions in proportions similar to the individual kinase activities, but the absolute binding activity was much lower than the control (Figure 1A). In separate experiments, the substrate proteins at the indicated concentrations were found not to interfere with either [^{3}H]cGMP binding or protein kinase assays of the enzyme preparation. These results indicate that some free catalytic subunit, as well as the intact native enzyme, was present in each eluate. Surprisingly, the free catalytic subunit derived in the histone and protamine experiments was not eluted from the affinity column by phosphate buffer. Therefore, we must conclude that the free catalytic subunit of cGMP-kinase is somehow also binding to blue dextran. Furthermore, the binding of dissociated subunits to blue dextran was tighter, since less [^{3}H]cGMP binding activity was detected in eluates, and a substantial amount of kinase activity was eluted with 1 M KCl. In addition, the very low binding and enzyme activity observed after histone preincubation suggested the instability of free subunits.

DISCUSSION

Blue dextran-Sepharose affinity chromatography has been used to purify many enzymes bearing nucleotide-specific allosteric binding sites or nucleotide substrate sites (15). They include phosphofructokinase (16), lactate dehydrogenase (8), and a catalytic subunit of cAMP-dependent protein kinase II (17). Unlike cAMP-kinase II, blue dextran binds to the native cGMP-kinase and competes with cGMP at the same binding site in the enzyme molecule However, like cAMP-kinase II, the ATP binding site of cGMP-kinase is not involved in the binding between native enzyme and blue dextran, since ATP + Mg^{++} neither prevents cGMP-kinase from binding to blue dextran-Sepharose column nor facilitates the elution of bound enzyme from the column.

Since substrate dissociates cGMP-kinase into subunits, a different chromatogram is expected when the enzyme preparation is incubated with histone and protamine prior to affinity column chromatography. The dissociated enzyme which was eluted should be composed of free subunits only. The reduction of [^{3}H]cGMP binding seems to confirm this notion. Furthermore, the finding that cGMP either did not activate kinase activity (histone), or it activated kinase activity to a much less degree (protamine) also supports this suggestion. Nevertheless, the fact that the catalytic subunit was eluted only by 0.2 M KCl, and not by phosphate buffer, should be explained by a second binding site between blue dextran and cGMP-kinase located at the catalytic subunit of the enzyme.

In conclusion, our findings support the "dissociation model" of cGMP-kinase proposed by Miyamoto et al. (11), Kuo et al. (18), and Van Leemput-Coutrez et al. (19).

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