AFFINITY CHROMATOGRAPHY ISOLATION AND CHARACTERIZATION OF SOLUBLE cGMP-BINDING PROTEINS FROM *AVENA SATIVA* L. SEEDLINGS

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Summary. In attempt to elucidate the early molecular events associated with the biological action of cyclic guanosine 3′,5′- monophosphate (cGMP) its binding to the structural components of *Avena sativa* L. plant cell has been studied. cGMP was shown to be bound specifically to proteins located predominantly in the soluble cytosolic fraction. cGMP-agarose affinity purification procedure followed by SDS-PAGE has revealed about ten specific cGMP-binding proteins in the cytosol. The nature of the purified proteins has been discussed..

Key words: *Avena sativa* L., affinity chromatography purification, cGMP, cGMP-binding activity, cGMP-binding proteins, soluble cytosolic fraction.

Abbreviations: cGMP – cyclic guanosine 3′,5′-monophosphate, cAMP – cyclic adenosine 3′,5′-monophosphate, cIMP – cyclic inosine 3′,5′- monophosphate, PDE – phosphodiesterase, NDPK – nucleoside diphosphate kinase, DTT - dithiothreitol, PMSF - phenylmethylsulfonyl fluoride

INTRODUCTION

Although cGMP is a well-known signaling molecule in both eukaryotes and prokaryotes, the unequivocal evidence for its occurrence and understanding of functions in higher plants is only recently emerging. cGMP was shown to play a crucial role in light, phytohormone and nitric oxide signal transduction in plants (Newton et al., 1999).

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The specificity of cellular responses to cGMP is based on the cGMP-binding activities of target proteins. Unfortunately, molecular targets of cGMP are poorly characterized even in mammalian cells. At present the elucidation of cGMP-binding activity and identification of cGMP targets in a plant cell are at an initial stage of investigation.

Two evolutionarily distinct allosteric sites for cGMP-binding are present in eukaryotic cells. One of them exhibits significant sequence homology with cGMP- dependent protein kinase and cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase and the cyclic nucleotide-gated cation channels, while the other one occurs in cGMP-regulated phosphodiesterases (PDE). Thus, cGMP can control cellular processes by means of protein phosphorylation or using nonphosphorylating mechanisms.

Three cyclic-nucleotide–dependent protein kinases have been reported in *Lemna paucicostata* (Kato et al., 1993). All kinases can phosphorylate histones. The first one is stimulated by cAMP, cGMP and cyclic inosine 3′,5′- monophosphate (cIMP), the second is inhibited by these mononucleotides, and the third is sensitive only to cGMP and cIMP. Phytochrome–associated protein kinase in rice leaves was reported to be activated by Ca^{2+} and cGMP (Komatsu and Hirano, 1993).

Recently, the biochemical evidence for soluble cGMP-regulated protein kinase in *Pharbitis nil* was obtained (Szmidt-Jaworska et al., 2003). From the conventional modulators only cyclic GMP, when applied at low concentrations, was able to stimulate the enzyme activity in the presence of histones. However, at the molecular level a cGMP-dependent protein kinase has not been identified so far.

cGMP-inhibited phosphatases of 30 kDa molecular mass were purified from silver beet leaves (K_d=3,3μM) and potato tubers (K_d=2,1μM) (Polya and Hunziker, 1987; Polya and Wettenhall, 1992). Plasma membrane H^+-ATPase was inhibited *in vitro* by cGMP in *Tradescantia* stem and leaf cells (Suwastika and Gehring, 1999).

The K^+-channels KAT1 (Anderson et al., 1992) and AKT1 (Sentenac et al., 1992) in *Arabidopsis* and the cation channel HvCVT-1 in barley aleurone layers (Schuurink et al., 1998) were shown to have a cGMP-binding site in the C-terminal region that is consistent with the involvement of cGMP in ion channel regulation. Ca^{2+}-dependent cyclic nucleotide-gated ion channels were found in plasma membranes isolated from barley aleurone (Schuurink et al., 1998), tobacco (Arazi et al., 2000) and *Arabidopsis* (Leng et al., 1999).

cGMP binds to allosteric sites, stimulates PDE activity and increases cGMP hydrolysis forming a negative-feedback mechanism for the regulation of cGMP concentration in cytoplasm. Similarly, cGMP enchances the PDE-mediated degradation of cAMP, e.g. cross-regulating its intracellular concentration that was demonstrated by the kinetic analysis of PDE isolated from *Lactuca* cotyledons (Chiatante et al., 1987).

Recently the evidence that cGMP modulates the expression of phytochrome-regulated genes of chalcone synthase (Arguello-Astorga and Herrera-Estrella, 1996) and

asparagine synthase (Neuhaus et al., 1997) and gibberellic acid-induced gene of α-amylase (Penson et al., 1996) was adduced.

Hence, the cGMP-binding activity of cellular components of higher plants is not sufficiently studied. Similarly to mammalian cells, the information on potential targets of cGMP action in plants like cGMP-regulated protein kinases, cGMP-gated ion channels and cGMP-regulated phosphodiesterases is available. However in plants other cGMP-regulated proteins are likely to occur and their identification is of great importance.

In this paper we report our attempts to reveal the targets for cGMP action in *Avena sativa* L. seedlings using the affinity purification and electrophoresis identification of cGMP-binding proteins that have not been previously demonstrated in higher plants.

MATERIALS AND METHODS

Plant material

Primordial leaves of 5-day-old oat seedlings (*Avena sativa* L. cv. Asilak) were used. The plants were grown at 20^0C under the conditions when 15 hr polychromatic white light (30 W m^{-2}) was alternated with 9 hr continuous dark.

Cell fractionation

The subcellular fractions were obtained using differential centrifugation as described (Volotovski et al., 2003). Briefly, the leaves were homogenized in a medium containing 50 mM Tris-HCl (pH 8.3), 0.3 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 2 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was filtered and clarified by centrifugation at 1500 g for 10 minutes. The crude mitochondrial fraction was pelleted by centrifugation of above supernatant at 10000 g for 10 minutes. To obtain a soluble cytosolic fraction this supernatant was centrifuged at 113000 g for 30 minutes. The pellet was resuspended and used as a crude microsomal fraction immediately after the separation. Plasma membranes were isolated from the crude microsomal fraction according to the two-phase method (Larsson et al., 1987). All procedures were performed at 4^0C.

Radioactive cGMP-binding assay

The binding assay was performed in 0.5 ml of the mixture containing 20 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, 0,1 mM EDTA, 2 mM dithiothreitol, 2 mM theophylline, 10^{-7} M [^{3}H]cGMP (396 TBq/mol) and 0,3 mg of protein. The mixture was incubated routinely for 60 minutes at 20^0C. The cGMP-binding to membranes was terminated by transferring the mixture to GF/F glass-fiber filter (Whatman, UK). The in-

cubation mixture containing the cytosolic fraction was transferred to BA 85 nitrocellulose filter (Schleicher & Schuell, Germany). The filters were washed with 12 ml of chilled 70 mM phosphate buffer (pH 7.0), air-dried at 70^0C and placed in liquid scintillation mixture OptiPhase 'HiSafe' 3 (Wallac Oy, Finland). The amount of bound cGMP was determined using Wallac 1409 liquid scintillation counter (Wallac Oy, Finland). As a reference a sample containing 10^{-5} M non-radioactive cGMP was used. All values were corrected for non-specific binding.

Soluble protein extraction for affinity chromatography

Oat leaves were homogenized with mortar and pestle in two volumes (g/ml) of medium containing buffer A (50 mM glycerophosphate (pH 7.4), 20 mM EDTA, 15 mM $MgCl_2$ and 5 mM NaF) with addition of 1% (w/v) polyvinylpolypyrrolidone, 1% (v/v) plant protease inhibitor cocktail (Sigma), 500 µM Na_3VO_4, 10 µM NH_4MoO_4 and 2 mM dithiothreitol. The homogenate was filtered and cleared by centrifugation at 100000 g for 30 minutes at 4^0C. The obtained supernatant was used as a fraction of soluble cytosolic proteins for affinity chromatography. The protein content was measured according to Bradford, (1976).

Affinity purification of cGMP-binding proteins

The extract containing soluble proteins (4.5 mg total protein) was applied to a column loaded with 600 µl of 8-(2-aminoethyl)thioguanosine-3',5'-cyclic monophosphate-agarose (8-AET-cGMP-Agarose) with 8-AET-cGMP being immobilized as an affinity ligand. The column was equilibrated with 6 ml of buffer A at 25^0C (100-200 µl/min). After washing with 30 ml of buffer A (200 µl/min) the bound proteins were eluted by incubation for 15 hours in buffer A supplemented with the competitive 10 mM cGMP. Eluted proteins were precipitated in 10 volumes of ice-cold acetone containing 10% (w/v) trichloroacetic acid and 0.07% (v/v) β-mercaptoethanol, washed twice with ice-cold acetone containing 0.07% (v/v) β-mercaptoethanol. After air-drying at 30^0C samples were incubated at 95^0C for 5 min in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0,01% bromophenol blue.

SDS-PAGE and silver staining of purified proteins

All fractions were analysed by one-dimensional electrophoresis on a 0.75 mm 10% acrylamide gel using the standard method of Laemmli (1970) followed by silver staining similar to Shevchenko et al., (1996). After electrophoresis, the slab gel was fixed in solution containing 40% ethanol and 10% acetic acid for 60 min. It was then washed twice for 10 min with 10% ethanol and additionally five times for 5 min with water to remove the remaining acid. The gel was sensitized by 2 min incubation in

solution containing 0.05% $K_3Fe(CN)_6$, 0.3% $Na_2S_2O_3$ and 0.5% Na_2CO_3 and was then rinsed by water for four times 5 min each. After rinsing, the gel was submerged in 0.1% $AgNO_3$ and incubated for 30 min. Then the silver nitrate solution was discarded, the gel slab was rinsed by 2.5% Na_2CO_3 for 5 min and then developed by 2.5% sodium carbonate containing 0.02% formaldehyde under intensive shaking. After the desired intensity of staining was achieved the development was terminated by discarding the reagent followed by washing of the gel slab with 5% acetic acid for 5 min. Silver stained gels were stored in 1% acetic acid at 4 ^{0}C. The kit of proteins with molecular weights between 14.4 and 116.0 κDa (Fermentas, Lithuania) was used as molecular weight standard marker.

Results and discussion

Previously we demonstrated a cGMP-binding activity in homogenate of *Avena sativa* L. seedlings (Dubovskaya et al., 2002). This binding appeared to depend on the incubation conditions and reagents concentration. The Scatchard plot analysis indicated the presence in *Avena sativa* L. cells of two classes of cyclic GMP-specific binding sites with high and low affinities for cGMP.

To determine the distribution of cGMP-binding activity in plant cells, oat seedlings were homogenized and subcellular fractionation was performed. Then the binding activity for cGMP in various fractions was evaluated. cGMP was found to bind predominantly to proteins in the soluble cytosolic fraction (Fig. 1). The concentration of the sites with low and high affinity for cGMP were 4.6 and 1.4 pmol/ mg protein with Kd=252.9 and 5.6 nM, respectively.

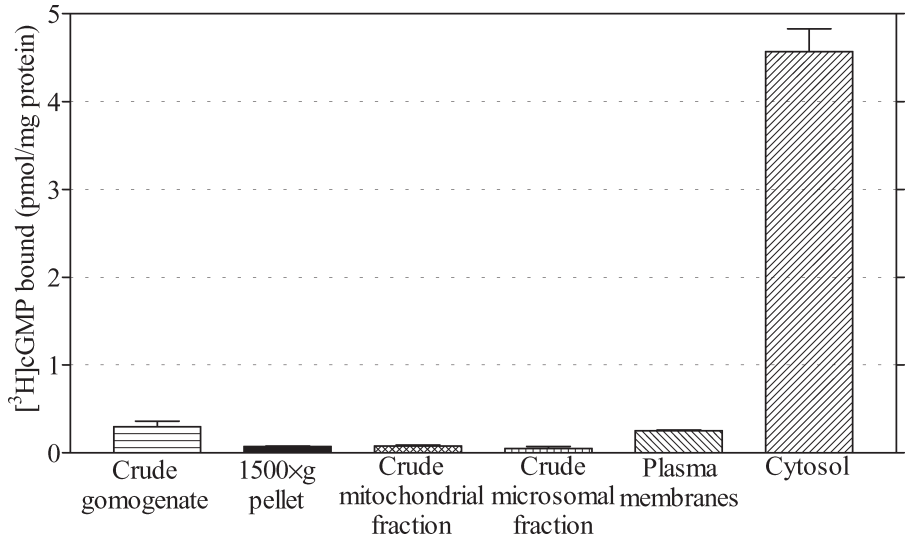

Fig. 1. Specific [^{3}H]cGMP-binding in subcellular fractions isolated from *Avena sativa* L. seedlings. The values are means ± SD (n=5).

Heating the cytosolic samples to 100^0C for 5 min completely suppressed the ability of the sites to bind to cGMP (Fig.2). Moreover, 70% of the binding activity was lost after the treatment of samples with trypsin (0.02 mg/ml) and pronase (1 mg/ml). The addition of soybean trypsin inhibitor to the incubation medium restored the binding activity. Together with the dependence of the binding activity on pH (Fig. 3) these results suggest a protein nature of the cGMP-binding sites.

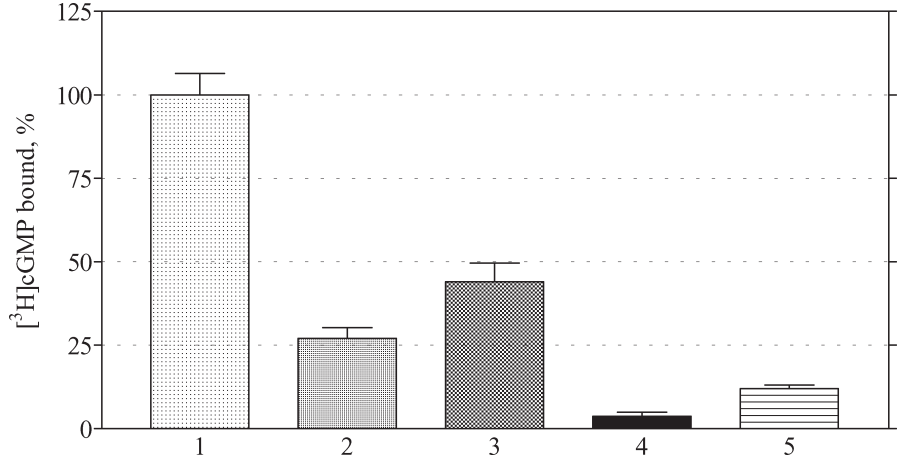

Fig. 2. Effect of trypsin (0,02 mg/ml), soybean trypsin inhibitor (0,08 mg/ml), pronase (1 mg/ml) and 100^0C heating on specific [^{3}H]cGMP-binding in the cytosol of cells of *Avena sativa* L. seedlings. 1 – control without treatment (100% binding), 2 – trypsin, 3 – trypsin in presence of soybean trypsin inhibitor, 4 – pronase, 5 – heating to 100^0C for 5 min. The values are means ± SD (n=5).

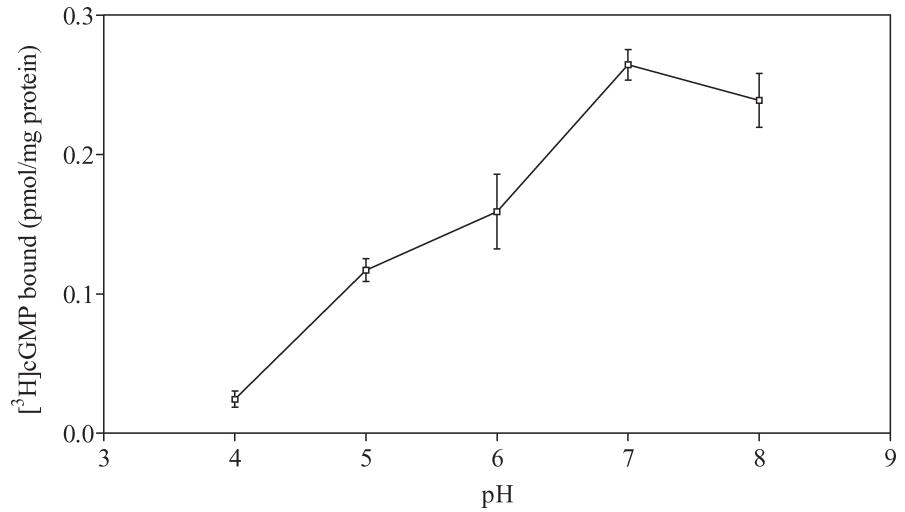

Fig. 3. Effect of pH on specific [^{3}H]cGMP-binding in crude homogenate of *Avena sativa* L. seedlings. The values are means ± SD (n=5).

Taking into account the occurrence of high cGMP-binding activity and the protein nature of the sites in the soluble cytosolic fraction, we made an attempt to characterize cGMP-binding proteins in the cytosol of oat cells. To purify cGMP-binding proteins we used affinity chromatography on 8-(2-aminoethyl) thioguanosine-3',5'-cyclic monophosphate-Agarose gel. Ten specifically bound proteins in the cytosol were eluted from the column with an excess of cGMP and identified by SDS-PAGE in cytosol (Fig. 4, lane 5). They showed an apparent molecular weight of 15 and 18 кDa, about 30-40 кDa and 53, 58 и 72 кDa. In experiments in which the protein purification was performed after preincubation of plant extract with 10 mM cGMP no proteins (Fig. 4, lane 4) were detected that confirms a specific interaction of the sites with cGMP.

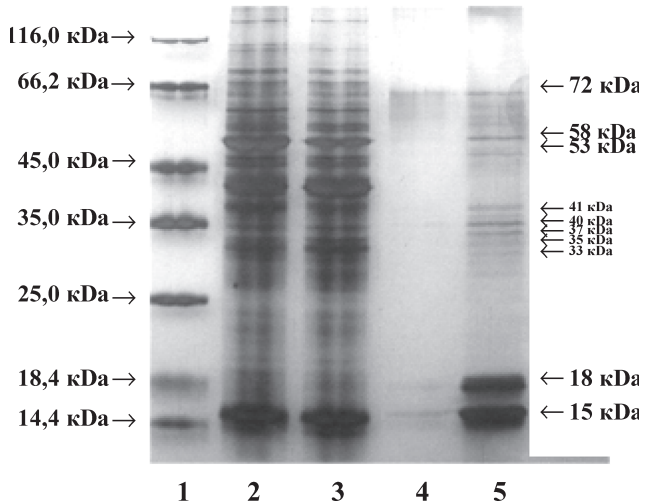

Fig. 4. 10% SDS-PAGE of cGMP affinity-purified *Avena sativa* L. proteins. Lane 1 – molecular weight marker, lane 2 – total protein in tissue homogenate, lane 3 – total protein in cytosol, lane 4 – cytosolic fraction eluted with 10 mM cGMP when cytosolic proteins were preincubated with 10 mM cGMP before chromatography (nonspecific binding), lane 5 – cytosolic fraction eluted with 10 mM cGMP. Positions of the molecular weight markers are shown on the left, estimated molecular masses of purified proteins are shown on the right.

Further, we suppose the probable nature of the detected protein bands taking into consideration the data available. The principal targets for cGMP in the cytosol are likely to be presented by cGMP-dependent protein kinases and PDEs. At least five cGMP-binding proteins (110, 80, 55, 49 and 38 kDa) identified as cGMP-inhibited cyclic AMP PDE (110 кDa), cGMP-dependent protein kinase (80 кDa) and two regulatory subunits of cAMP-dependent protein kinase (49 и 55 кDa) were

detected in the cytosol of human platelets by photoaffinity labeling (Tang et al., 1993). Two soluble proteins of 93 and 72 kDa, corresponding respectively to the alpha subunit of cGMP PDE and cGMP-dependent protein kinase were detected in bovine retina (Thompson and Khorana, 1990).

A soluble protein kinase was isolated and purified from seedlings of morning glory *Pharbitis nil* by anion-exchange and affinity-chromatography. The enzyme activity was regulated by cGMP and consisted of a single 70 kDa polypeptide (Szmidt-Jaworska et al., 2003). We suppose that the 70 kDa cGMP-binding protein found in the cells of *Avena sativa* L. is likely to be a cGMP-dependent protein kinase.

Chiatante et.al. (1988) performed a partial immunoaffinity purification of multifunctional cyclic nucleotide PDE from *Lactuca* cotyledons. The enzyme contained several catalytic sites regulated by each other. The molecular weight of the protein was determined as 62 кDa although all mammalian cGMP-binding PDEs are known to have mainly molecular weight exceeding 80 kDa. Only Ca^{2+}-calmodulin-dependent PDEs, which bind cGMP in the catalytic site possess subunits of 58-75 kDa. It is tempting to suggest that the isolated proteins with molecular weight of 53 and 58 kDa are PDE monomers containing cGMP-binding sites.

Another group of the detected proteins with molecular weight of 30-40 kDa is similar to cGMP-inhibited phosphatases of 30 kDa purified from silver beet and potato (Polya and Hunziker, 1987; Polya and Wettenhall, 1992).

Of particular interest among the purified cGMP-binding proteins are the low-molecular weight peptides of 15 and 18 кDa which bind cGMP most efficiently. It should be noted that recently specific binding of cyclic AMP to similar soluble proteins in tobacco bright yellow 2 cells was shown (Laukens et al., 2001). These proteins were identified by mass spectrometry as two nucleoside diphosphate kinases (NDPKs). The analysed peptides from the 18 kDa band were 100% identical to *Pisum sativum* mitochondrial NDPK. The sequenced peptides from the 15 kDa band appeared to be 100% identical to *Capsicum annuum* NDPK. Plant NDPK isoforms are known to involve in phytochrome signaling (Choi et al., 1999), where they seem to act as a positive transcription factor (Zimmermann et al., 1999), and in stress signaling mediating heat response (Escobar Galvis et al., 2000). Since recent evidence shows that cGMP can act primarily via modulation of expression of light-regulated genes (Arguello-Astorga and Herrera-Estrella, 1996; Neuhaus et al., 1997), molecular targets for cGMP action in light signal transduction in plants are likely to be identified as NDPK isoforms.

In spite of the fact that soluble cGMP-binding proteins are purified and electrophoreticaly characterized, future investigation will be concentrated to elucidate the actual nature and full sequences of the detected proteins by means of mass

spectrometry and cloning strategy. It is then that the rapid one-step purification technique provided in this paper will be an appropriate tool for isolation and characterization of these proteins.

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