Volume 335, number 1, 81–84 FEBS 13298 November 1993

Enzymes of the cyclic GMP metabolism in bovine retina

I. Cloning and expression of the gene for guanylate kinase

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Received 30 September 1993

Guanylate kinase (EC 2.7.4.8) catalyzing the reaction GMP + ATP = GDP + ADP, was purified to homogeneity from bovine retina. Using oligonucleotides based on the amino acid sequence of this enzyme, the cDNA encoding guanylate kinase (GK) was isolated and its nucleotide sequence was determined. Expression of the GK cDNA in *E. coli*, and the purification and functional characterization of the expressed enzyme are presented. It is shown that bovine retinal GK, like its yeast counterpart, contains the characteristic glycine-rich motif and all the amino acids involved in GMP binding. Bovine retinal enzyme is extended for several amino acid residues both at the N- and C-termini, compared to the yeast enzyme.

cGMP cycle; Guanylate kinase; cDNA cloning; Expression; *B. taurus*

1. INTRODUCTION

Intensive studies on the nature of second messengers in visual excitation culminated in the discovery that cyclic GMP (cGMP) directly influences the conductance of the rod outer segment (ROS) plasma membrane [1,2]. Since then the metabolism of cGMP in the photoreceptor cell became a very important issue.

The enzymatic cascade that leads to visual excitation is now well documented. This cascade is initiated by the photoisomerization of the retinal chromophore of rhodopsin. One of the spectral intermediates of rhodopsin bleaching, metarhodopsin II, activates a GTP-binding protein, which, in turn, releases an inhibitory constraint from the effector enzyme (cGMP phosphodiesterase) resulting in hydrolysis of cGMP [3,4].

cGMP is formed from GTP by calcium-dependent guanylate cyclase [5,6]. The first step in this metabolic pathway is phosphorylation of 5′-GMP to GDP by guanylate kinase (GK). GDP is further phosphorylated by nucleoside disphosphate kinase to GTP. These two consecutive phosphotransferase reactions regulate the supply of guanine nucleotides not only to signal transduction pathways but also to protein synthesis, vesicular traffic, and cellular proliferation processes [7]. The renewed interest in these kinases, long considered as 'housekeeping' enzymes, came from the recent identification of the gene products of the *dlg* and *nm23* genes which are probably involved in neoplastic overgrowth of the imaginal discs in *Drosophila* [8,9] and mammalian tumor malignancy [10], respectively.

In the present report we present data on the purification, cloning and expression of bovine retinal GK involved in the cGMP cycle (cGMP → GMP → GDP → GTP → cGMP). Surprisingly, this is the first report on the sequence analysis of a GK gene from animal tissue.

2. MATERIALS AND METHODS

2.1. *Assay of GK activity*

Enzymate activities of retinal extracts and purified GK were measured by spectrophotometric assays according to [11].

2.2. *Preparation of GK*

GK was purified from bovine retinas as described [12], with minor modifications. Protein concentration was determined as in [13]. SDS-PAGE was carried out according to Laemmli [14].

2.3. *Preparation of tryptic peptides*

Purified GK was cleaved with trypsin with an enzyme:substrate ratio of 1:30 for 24 h at 37°C. The peptides were purified by two cycles of HPLC (Zorbax C18) on an Altex (model 332) system. The column was developed with a linear gradient of acetonitrile (0–70%) in 0.1% CF_3COOH. The peptides were sequenced by Edman degradation on a gas-phase sequencer (Applied Biosystems).

2.4. *Isolation and sequencing of cDNA clones*

The cDNA library of bovine retina in bacteriophage λ-ZAP was provided by Dr. M. Applebury (Department of Ophthalmology, University of Chicago). The isolation of cDNA clones was carried out as described in [15]. Sequencing of the inserts was performed by the

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The nucleotide sequence reported in this paper has been submitted to the EMBL Data Bank under accession number X67029.

Sequenase version [16] of the Sanger dideoxy chain termination method.

2.5. *Expression of GK as a fusion protein*

The cDNA of GK in plasmid pBL8 was subjected to site-directed mutagenesis to introduce a *Nco*I restriction site, which resulted in plasmid pBL8M. For expression of the GK cDNA a system described in [18,19] was used. The expression plasmid, pL2GK, was prepared by ligation of the pBL8M insert (*Nco*I–*Eco*RI) with vector pL2P26 (*Nco*I–*Eco*RI) [19], and then transformed into the *E. coli* strain, MH-1. For expression of fusion protein, cells were grown as recommended [19].

2.6. *Purification of the fusion protein*

Fusion protein was purified from culture supernatant by affinity chromatography on an IgG–Sepharose 6FF column, as described in [19].

2.7. *Cleavage of the fusion protein with enteropeptidase*

Purified fusion protein was subjected to enteropeptidase digestion as in [20]. GK and the protein A fragment were separated by affinity chromatography on IgG–Sepharose 6FF.

3. RESULTS AND DISCUSSION

GK (ATP:GMP phosphotransferase EC 2.7.4.8) is a soluble protein dispersed throughout the cytosol of retinal photoreceptor cells [21,22]. The enzyme was purified both from isolated ROS and entire retina, and the identity of both samples was shown [12]. The enzyme was shown to be extractable under hypotonic and isotonic conditions, as well as from light- and dark-adapted ROS [12].

The purification procedure of GK presented in this paper was based on that in [12] with minor modifications. The yield of GK was about 90 U from 100 retinas, and the specific activity of the purified enzyme was 330 U/mg of protein. In SDS-PAGE the purified enzyme migrated as a single band with a molecular mass of 22 kDa (see Fig. 4).

It was shown earlier that in the living retina the turnover of cGMP occurs at a half-life of only 1.5 s and this rapid turnover is even accelerated 5-fold upon illumination [23]. Thus, the presence of GK with an activity comparable to cGMP phosphodiesterase in the ROS is an important factor in maintaining the rapid turnover of cGMP.

Direct sequence analysis of the intact protein did not show cleavage of any amino acids, indicating that the N-terminus is blocked. The nature of this blocking group in retinal GK remains to be determined. Digestion with trypsin followed by separation of peptides using RP-HPLC on Zorbax C_{18} resulted in considerable sequence information accounting for 50% of the polypeptide chain (see Fig. 2 underlined). Using this information and taking into account the codon usage of several known ROS proteins, two oligonucleotide probes shown below were prepared to screen the retinal cDNA library.

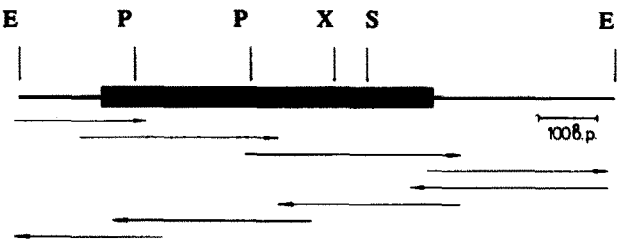

Fig. 1. Restriction map and sequence strategy used in the analysis of the GK cDNA. The solid box indicates the coding region. Arrows show the extent and direction of sequence determination. E = *Eco*RI, P = *Pst*I, S = *Stu*I, X = *Xma*I.

Peptide	EENGKDYYFV
Oligonucleotide	5′ GAGGAGAACGGCAAGGACTACTTCGT 3′
Peptide	DFIEHAEF
Oligonucleotide	5′ GACTTCATCGAGCACGCGGAGTT 3′

Seven clones were isolated from screening of 10^6 transformants. One of the largest clones (pBL8) was selected for further analysis (Fig. 1). The nucleotide sequence of its cDNA insert is presented on Fig. 2. The open reading frame between bases 184 and 758 appears to contain the entire coding sequence of GK. The de-

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  I  GTGCACTTCTCGGGTGAGGAGCAGCGTTTCCCTCTGTTGAGCAGGTGGTGCGGGCTGCAC   60
 6I  TAGGAGGGATTTAGTGCTCAGACAGTGGGGAGCAGGCAAGGATTACCACTGTCTCAGCTC  I20
I2I  AGCCAGTGCAGCCCCAAGCTGAGCACAGAGGCTACTCAGAAGGGATGTCAGGACCAAGGC  I80
                                                  M  S  G  P  R  P

I8I  CCGTTGTCCTGAGCGGACCCTCAGGGGCTGGGAAGAGCACCCTACTGAAGAAACTCCTGC  240
       V  V  L  S  G  P  S  G  A  G  K  S  T  L  L  K  K  L  L  Q

24I  AGGAACATGGCAGCATCTTTGGCTTCAGCGTGTCCCACACGACAAGGGACCCGAGGCCAG  300
       E  H  G  S  I  F  G  F  S  V  S  H  T  T  R  D  P  R  P  G

30I  GAGAGGAGAACGGCAAAGATTACTACTTTGTGACCAGGGAGGTGATGCAGCGCGACATTG  360
       E  E  N  G  K  D  Y  Y  F  V  T  R  E  V  M  Q  R  D  I  A

36I  CTGCTGGAGACTTCATCGAGCACGCTGAGTTCTCAGGGAACTTGTATGGGACCAGCAAGG  420
       A  G  D  F  I  E  H  A  E  F  S  G  N  L  Y  G  T  S  K  A

42I  CCGCCGTGCGGGCCGTGCAGGCCATGAACCGCATCTGCGTGCTGGACGTGGACCTGCAGG  480
       A  V  R  A  V  Q  A  M  N  R  I  C  V  L  D  V  D  L  Q  G

48I  GCGTGCGCAACATCAAGAAGACCGACCTGCGGCCCATCTACATCTTCGTGCAGCCGCCCT  540
       V  R  N  I  K  K  T  D  L  R  P  I  Y  I  F  V  Q  P  P  S

54I  CACTGGATGTCCTGGAGCAGCGGCTGCGACAGCGGAACACAGAGACAGAGGAGAGCCTGG  600
       L  D  V  L  E  Q  R  L  R  Q  R  N  T  E  T  E  E  S  L  A

60I  CCAAGCGTCTGGCGGCTGCCCGGGCTGACATGGAGAGCAGCAAGGAGCCCGGCCTGTTTG  660
       K  R  L  A  A  A  R  A  D  M  E  S  S  K  E  P  G  L  F  D

66I  ACCTGATCATCGTCAACGACAGTCTGGACAAGGCCTACTGGGCCCTGAAGGAGGCGCTCT  720
       L  I  I  V  N  D  S  L  D  K  A  Y  W  A  L  K  E  A  L  S

72I  CCGAGGAAATAAAGAAGGCCCAAGGCACTGGTCAGTCCTGAGGAGGCCCGCCAGCTGTGT  780
       E  E  I  K  K  A  Q  G  T  G  Q  S  Ter

78I  CCTCTCAGGTGGGGCCCAGGGCCTGGCGCCCACGTCAGTGGGCCAGAGTTTCCTGGCAGT  840
84I  GACTCTGCTCACCTCAGCTGGGACCCAGAGACAGGGCGCTGCCCATCTTCCCTCATCCCC  900
90I  TCCGTGCTTGGAGGACCTTGTCTCTTACCCCCATGCCCCACGTCCATTTCTAGGCTTCCT  960
96I  CCCCACCATGCCCTGTCAACCCCTTACCCGTGGAAGCCAGGCCAACATCCAAATAAAGAA  I020
I02I CTGCTGGGTTAAAAAAAAAAAA I042
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Fig. 2. Nucleotide sequence of GK cDNA and deduced amino acid sequence. The sequences determined by peptide analysis as well as polyadenylation signal are underlined. Ter = translation termination signal.

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          10v        20v        30v        40v        50v
RETINA  MSGPRPVVLSGPSGAGKSTLLKKLLQEHGSIFGFSVSHTTRDPRPGEENGK
          .::.:.::::::.:::::::::  :    :::::: ::: ::.:: :::
YEAST     SRPIVISGPSGTGKSTLLKKLFAEYPDSFGFSVSSTTRTPRAGEVNGK
               10^        20^        30^        40^
           60v        70v        80v        90v        100v
RETINA  DYYFVTREVMQRDIAAGDFIEHAEFSGNLYGTSKAAVRAVQAMNRICVLDV
          :: ::. .  .  :   .::: :.::::: ::.. :.:. :    . :.::.
YEAST   DYNFVSVDEFKSMIKNNEFIEWAQFSGNYYGSTVASVKQVSKSGKTCILDI
          ^        60^        70^        80^        90^
             110v       120v       130v       140v       150v
RETINA  DLQGVRNIK-KTDLRPIYIFVQPPSLDVLEQRLRQRNTETEESLAKRLAAA
          :.:::. .:   .: . ..:. :::.. :  ::  : :::::::. :::.::
YEAST   DMQGVKSVKAIPELNARFLFIAPPSVEDLKKRLEGRGTETEESINKRLSAA
          ^       110^       120^       130^       140^       150^
              160v       170v       180v       190v
RETINA  RADMESSKEPGLFDLIIVNDSLDKAYWALKEALSEEEKKAQGTGQS
          :..  . : :  : .:::: :::::  ::. .  :
YEAST   QAELAYA-ETGAHDKVIVNDDLDKAYKELKDFIFAEK
                160^       170^       180^
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Fig. 3. Comparison of amino acid sequences between bovine retinal and yeast GK. (:) identical amino acid; (.) homologous amino acid.

duced sequence of GK consists of 198 amino acids with a calculated molecular mass 22,051 Da.

During the last two decades much preference was given to studies of adenylate kinase from different sources. These efforts resulted in several X-ray structures [24,25]. Much less is known about GKs. The only amino acid sequence and high resolution X-ray structure known is that of the yeast enzyme [26–28]. Comparison of the amino acid sequences of the GK from bovine retina and from yeast shows 55% identity. The amino acid sequence of bovine retinal GK is extended at the N- and C-termini for 2 and 9 residues, respectively, compared to the yeast enzyme (Fig. 3).

Refined X-ray structure of the complex between the yeast GK with bound GMP at 2.0 Å resolution clearly shows that Arg-38, Arg-41, Tyr-50 and Tyr-78 are involved in the hydrogen bonding of a phosphate group [28]; all these amino acids, together with Glu-44 which is salt-bridged to Arg-38, are conserved in the retinal counterpart (see Fig. 3). Also conserved are the amino acids involved in the binding of the purine ring of GMP via non-polar interactions or hydrogen bonding: Ile-99, Tyr-78, Glu-69 and Ser-80, as well as Asp-98, contributing to the binding of the magnesium ion in the adenylate kinase [29]. The catalytic cleft of yeast GK was shown to contain several arginine residues (Arg-131, Arg-135, Arg-146) which are likely to fix the phosphate groups during phosphoryl transfer [28]. All these arginine residues are conserved in the retinal enzyme. The threonine residue in the putative ATP-binding glycine-rich motif (residues 11–17) of the yeast enzyme is substituted for alanine in the retinal counterpart.

As a first step in the structure–functional studies of GK we expressed it in *E. coli* as a fusion protein. It was shown earlier that vectors containing the sequence of protein A from *Staphylococcus aureus* can successfully be used for the expression of different fusion proteins and peptides [17]. Moreover, a synthetically modified fragment encoding the IgG-binding site of protein A was shown to be as effective as the whole gene. This synthetic fragment was chosen to meet *E. coli* codon preference and contained a signal sequence for the secretion of a fusion protein [18]. The expression plasmid for GK was constructed based on the plasmid, pL2P26, described earlier [19]. As the GK cDNA does not contain any suitable restriction sites for cloning of the full-size coding region into the expression plasmid, an *Nco*I site was introduced into the region of the initiating codon using the following oligonucleotide: 5′ CTACTCAGAAGCCATGGCAGGACCA3′. This mutation also substituted Ser-2 for Ala. The expression construct based on the mutant plasmid, pBL8M, and expression plasmid, pL2P26 [19], was transformed into *E. coli*. To facilitate the secretion of the fusion protein the culture was grown in the presence of IPTG under vigorous aeration at the heat-shock temperature. According to SDS-PAGE, the fusion protein, as expected, was intensively secreted from cells and mostly present in the supernatant. Purification of the recombinant fusion protein using affinity chromatography on IgG– Sepharose yielded 5 mg of the protein per 1 l of supernatant. The fusion protein was further subjected to enteropeptidase cleavage at the Lys–Ala bond of the peptide (Asp)4-Lys-Ala, adapted to the N-terminus of GK, followed by the final purification on IgG–Sepharose [19,20]. Native and recombinant enzymes exhibited indistinguishable electrophoretic mobilities on SDS- PAGE (Fig. 4). This fact, together with the specific binding to Blue–Sepharose and the absolute requirement of ATP and GMP as donor and acceptor of the phosphate group, respectively, indicates that the purified recombinant GK has the native conformation (data not shown). In addition, the recombinant GK exhibits a specific activity close to that of the enzyme from retina.

The nucleotide sequence of GK cDNA and its successful expression presented in this work (see also [27]), taken together with available high resolution X-ray

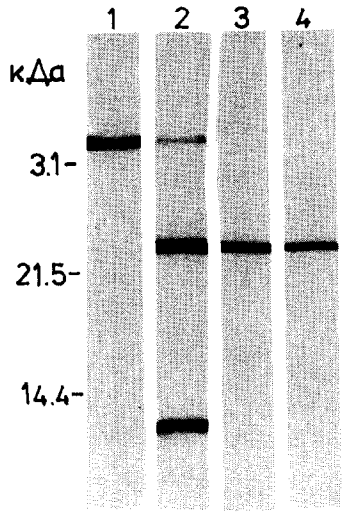

Fig. 4. SDS-PAGE of recombinant GK. SDS-PAGE was carried out in 15% gels. Lane 1, IgG–Sepharose- and Blue–Sepharose-purified fusion protein; lane 2, the fusion protein treated with enteropeptidase (20 mM Tris-HCl, pH 8.0, 37°C, 4 h); lane 3, IgG–Sepharose-purified expressed GK; lane 4, GK from bovine retina.

structural data on the yeast enzyme [28], provide possibilities for further studies of phosphotransferase reactions with the combined use of biophysical and molecular biological techniques.

As GK catalyzes the first step in the recovery of cGMP (GMP → GDP → GTP → cGMP) its function is crucial for maintaining a certain level of guanine nucleotides in the photoreceptor cells. In this respect it would be interesting to see whether any of the known disorders in these cells are connected with abnormalities in the functioning of GK. Recently it was shown that the discs large tumor suppressor gene, *dlg*, of *Drosophila*, mutation of which causes neoplastic overgrowth of imaginal discs, incorporates a DNA fragment encoding a protein highly homologous to yeast GK [8,9]. It was concluded that the tumor suppressor action of *dlg* might be due to its involvement in the control of GDP and GTP levels, thus regulating the activity of p21ras or other GTP-binding proteins. Furthermore, the major palmytoilated membrane protein from human erythrocytes was also found to display high homology to yeast GK [9]. Despite the remarkable homology, neither of these proteins were shown to display the GK activity. In this respect it would be interesting to see whether retinal GK with a disrupted glycine-rich motif is enzymatically active. It remains to be determined whether GK, which has been thought to be one of the 'housekeeping' enzymes of the cell, also functions as a tumor suppressor.

Acknowledgements: We thank Dr. Ingrid Albert (Max-Planck Institute of Biophysics, Frankfurt/Main, Germany) for careful reading of the manuscript, and Dr. Boris Shmukler for helpful discussions.

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