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Expression of mouse rod photoreceptor cGMP phosphodiesterase γ subunit in bacteria

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We expressed the γ subunit of mouse rod photoreceptor cGMP phosphodiesterase (PDE) in the bacterial pGEX-2TK expression vector which produces a cleavable 40 kDa fusion protein. The fusion protein can be isolated in a one step procedure by affinity chromatography on glutathione beads. The yield of purified fusion protein is approximately 10 mg from 1 liter of bacterial culture, or about 3 mg of PDEγ, equivalent to the PDEγ content of approximately 200,000 mouse retinas. Both the fusion protein and the cleaved PDEγ, to which a short kinase domain remains attached, are biologically active, inhibiting activated PDE in a manner comparable to native PDEγ. Immobilized PDEγ binds transducin α subunit charged with GTP, PDE α and β subunits, and, unexpectedly, arrestin (S-antigen).

Mammalian phototransduction; Bacterial expression; cGMP phosphodiesterase γ subunit

1. INTRODUCTION

Rod photoreceptor cGMP phosphodiesterase (PDE) consists of 3 subunits termed α, β, and γ according to their apparent sizes on SDS gels [1]. The two large subunits α and β contain the active site(s) of the enzyme, the small subunit, PDEγ, is a heat-stable polypeptide acting as a inhibitor of activated PDE [2]. PDEγ cDNA was cloned from bovine [3], mouse [4], and human [5] retinal libraries and shown to consist of 87 highly conserved residues. PDEγ is hydrophilic (24 of its 87 residues are charged amino acids). The N-terminal half contains all 13 positive charges, while the C-terminal half is predominantly negatively charged. Mouse PDEγ differs from its bovine counterpart by two uncharged residues at position 8 and 17 ($G_{mouse}8A_{bovine}$ and $I_{mouse}17M_{bovine}$) which do not influence biological properties [6]. Apart from N-terminal acetylation [3] no other post-translational processing has been shown to occur. Bovine PDEγ was expressed in bacteria using a synthetic template in a vector which produced a biologically active, cleavable fusion protein consisting of 7 amino acid residues of the C-terminal end of the clotting protease factor Xa [7] and PDEγ (cIIγ). Mouse PDEγ was expressed as a noncleavable fusion protein (βgalγ) in the bacterial vector pUC19 [6]. Both procedures are relatively tedious requiring column chromatography for purification of the respective fusion proteins. Moreover, transformed bacterial strains expressing cIIγ are unstable [8].

As part of our effort to express an active mouse PDE holoenzyme in bacteria [9] or insect cells infected with recombinant baculovirus [10], we have cloned mouse PDEγ cDNA into the expression vector pGEX-2TK [11]. This vector construct produces a large fusion protein (40 kDa) consisting of 26 kDa of the C-terminal end of glutathione-S-transferase (GST), a thrombin cleavage site, an adapter (K) suitable for phosphorylation by protein kinases, and the 87 residues of mouse PDEγ. The expressed fusion protein, termed GK-PDEγ, can be rapidly isolated in large amounts by affinity chromatography on glutathione beads, and cleaved by thrombin to produce K-PDEγ (PDEγ to which the kinase domain remains attached). In this paper we describe the expression and isolation of GK-PDEγ (40 kDa) and K-PDEγ (14kDa) and show that both products are biologically active.

2. MATERIALS AND METHODS

2.1. *Expression vector*

Mouse PDEγ cDNA was amplified with PfuI thermostable DNA polymerase (Stratagene) on first strand retinal cDNA template using a N-terminal sense primer 5′-AAT **TGG ATC** CCT CAT GAA CCT GGA GCC ACC CAA GGG TG and a C-terminal antisense primer 5′-AAT **GGA TCC** GAG CTC TAG ATG ATG CCG TAC TGG GCC The primers include translation start and stop codons (underlined) of PDEγ and introduce *Bam*HI cloning sites (bold-faced). A 278 bp *Bam*HI fragment was cloned into pBluescript and completely sequenced. The DNA sequence was identical to the published sequence [4] except for codon 35 (ACA vs. ACC) which does not alter the predicted amino acid sequence. The 278 bp *Bam*HI fragment was filled in with dNTP/Klenow and cloned into the filled-in *Bam*HI site of the expression vector pGEX-2TK (generous gift of Dr. W. Kaelin, Dana

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Farber Cancer Center, Boston, MA). The correctness of the reading frame was confirmed by DNA sequencing with PDEγ specific primers. The recombinant plasmid, pGEX-2TKγ, was transformed into BL21DE3 strain (Novagen), and several transformants grown in overnight cultures consisting of 50 μg/ml ampicillin in LB broth.

2.2. *Isolation of GK-PDEγ and K-PDEγ*

The fusion protein was isolated by a procedure described by Smith and Johnson [11], as modified by Kaelin [12]. Briefly, an overnight culture was diluted 10-fold with LB/Amp, grown for an additional hour, and induced with 0.2 mM IPTG for two hours. Bacteria were spun down at 5 000 rpm/10 min (GSA rotor, sorvall), suspended in NETN buffer (0.5% NP-40, 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 100 mM NaCl), lysed by sonication, and the lysed cells centrifuged at 12 000 rpm/20 min (SA600 rotor, Sorvall). The insoluble pellet was treated with 8 M urea at room temperature for 30 min, and the solution dialyzed against lysis buffer. The dialysate was incubated with glutathione agarose beads (Sigma) for 30 min. The fusion protein was released from the beads by 2 treatments with 5 bead volumes each of 20 mM glutathione in 50 mM Tris-HCl pH 8.0 and 100 mM NaCl (30 min/4°C). The eluate containing the fusion protein was dialyzed against 50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 50% glycerol, and stored at −20°C. Protein concentrations were determined according to Bradford [13]. K-PDEγ was isolated by incubating 10 μg GK-PDEγ with 2 μl of human thrombin (Sigma, approx. 1 U/μl protein) in 50 mM Tris pH7 0, 150 mM NaCl, 2.5 mM $CaCl_2$ at room temperature for 1 h. The digestion was followed by boiling for 4 min and centrifugation (10 min, 15 000 × *g*). The supernatant, containing K-PDEγ was used for SDS-PAGE and PDE inhibition.

2.3. *Binding retinal polypeptides to immobilized GK-PDEγ*

Two bovine retinas (bleached) were homogenized twice in 2 ml each of hypotonic buffer (20 mM Tris, 0.1 mM DTT, 0.1 mM EDTA) containing GTPγS (2 μM) and PMSF (0.5 μM). The homogenates were spun at 15 000 × *g* for 15 min, and the supernatants were combined. To 200 μl of supernatant, 25 μl of 10 × isotonic buffer [1] and 20 μl glutathione agarose beads charged with GK-PDEγ were added The suspension was kept at 4°C for 30 min, spun at 3 000 ×*g* for 2 min, the supernatant discarded and the beads washed with 1 ml isotonic buffer three times. To the final washed pellet, 20 μl SDS sample buffer was added, mixed, and a 5 μl aliquot was used for SDS-PAGE.

2.4. *PDE assays*

PDE activity was assayed [14] by recording the release of protons with a pH microelectrode (MI-410, Microelectrodes, Inc.). The pH assay buffer contained 20 mM MOPS pH 8.0, 0.15 M KCl, 2 mM $MgCl_2$. cGMP was added to final concentration of 2 mM [15], the final volume was 200 μl.

2.5. *Isolation of transducin subunits, PDE, arrestin*

Transducin a subunit was eluted from ROS membranes in the presence of 2 μM GTPγS in isotonic buffer. Bovine PDE was isolated as described earlier [16]. Purified arrestin, a generous gift of Dr. Abdallah J. Ghalayini (Baylor College of Medicine), was isolated according to Wilden et al. [17,18].

3. RESULTS AND DISCUSSION

3.1. *Production and Isolation of GK-PDEγ and K-PDEγ*

PDEγ is present in rod photoreceptors in minute amounts, tightly bound to the PDE large subunits with a K_D (measured in bovine) of less than 10 pM [15]. To facilitate its isolation from bacterial cultures, we expressed mouse PDEγ as a fusion protein using the GST-2KT expression vector [11] (Fig. 1). This vector provides an N-terminal GST domain for rapid isolation of the product by affinity chromatography, a thrombin cleavage site (T) which allows removal of GST, and a kinase domain (K) which may be used to phosphorylate and label PDEγ. Induced by IPTG, the fusion protein is overexpressed and accumulates to about 10 mg/ liter of bacterial culture (Fig. 2, lane 4), corresponding to about 3 mg of K-PDEγ. Most of the fusion protein is stored in insoluble inclusion bodies (Fig. 2, lane 6), from which it may be released by treatment with 8 M urea (lane 7). The purified fusion product GK-PDEγ (lane 9), after digestion with thrombin, is cleaved into the GST component and K-PDEγ (lane 10). Taking advantage of the heat stability of PDEγ, K-PDEγ may be isolated from this mixture in purified form after a short boiling step and centrifugation (lane 11). K-PDEγ is

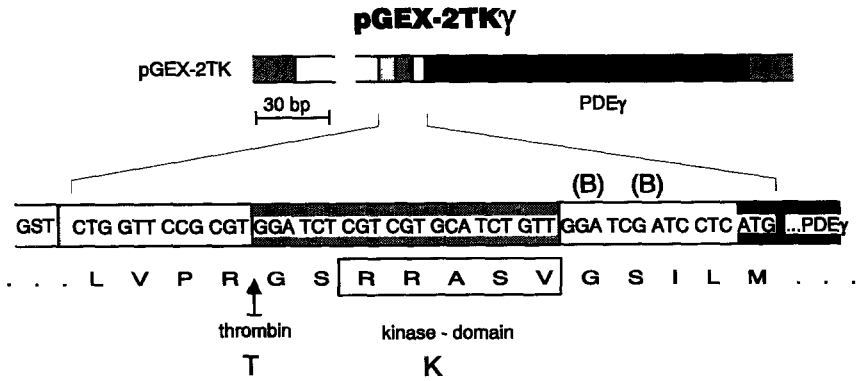

Fig. 1. Schematic representation of the expression vector construct. Upper part: schematic of the expression vector pGEX-2TKγ. The interrupted white box symbolizes the 26 kDa GST fragment, the stippled boxes depict the adapter containing the thrombin cleavage and protein kinase domain. PDEγ cDNA is shown as a large black box, and the cloning vector, pGEX-2TK, as open ended hatched boxes. Lower part: DNA and amino acid sequences of the adapter between the GST leader and PDEγ. The thrombin cleavage site (T) is marked by an arrow, and the kinase domain (K) is boxed. The 4 amino acid residues GSIL between the kinase domain and PDEγ are the result of in-frame cloning (ligation of two blunt-ended *Bam*HI sites, identified as (B) and underlined).

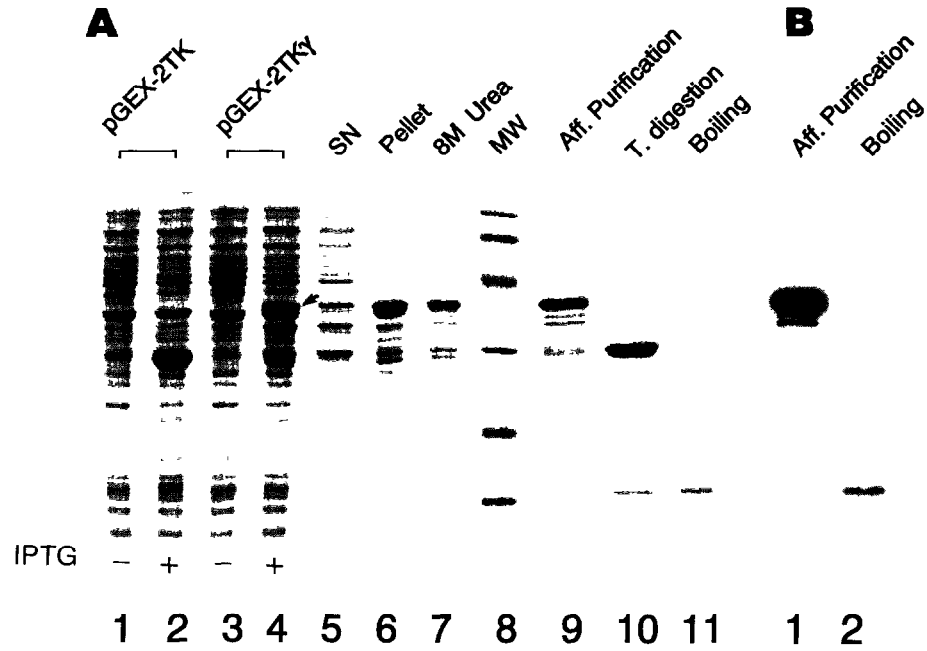

Fig. 2. SDS-PAGE/Western blot of purification of GK-PDEγ and K-PDEγ . A, stages of purification of GK-PDEγ. Lane 1 and 2, proteins expressed by the expression vector pGEX-2TKγ, before (−) and after (+) induction with IPTG, respectively. Lane 3, 4: proteins expressed by pGEX-2TKγ, before (−) and after (+) induction. The arrow points to the GK-PDEγ fusion protein (40 kDa). Lane 5, supernatant, and lane 6, pellet of a 100 ml bacterial suspension lysed in SDS buffer. Lane 7, pellet after solubilization in 8 M urea. Lane 8, molecular weight markers (see Fig. 5, lane 4). Lane 9, material bound to glutathione beads. Lane 10, the fusion protein GK-PDEγ eluted from the beads and cleaved with thrombin. The upper band is the GST leader, the lower band is K-PDEγ . Lane 11, purified K-PDEγ after boiling of the cleaved material in lane 10 for 4 min. B, Western blot. Lane 1, GK-PDEγ (lane 9 of A) product Lane 2, K-PDEγ (lane 11 of A).

stable in a solution of 50% glycerol at −20°C for at least one month.

3.2. *GK-PDEγ and K-PDEγ inhibit activated cGMP PDE*

Activation of PDE holoenzyme ($\alpha\beta\gamma_2$) in vivo is achieved by the transducin a subunit charged with GTP, T_α^{GTP} [19], involving physical removal or dislocation of PDEγ by a still unknown mechanism [8,20]. Limited trypsinolysis of PDE holoenzyme destroys the inhibitory PDEγ subunit, and generates a highly active PDE core enzyme PDEαβ in vitro [2]. To demonstrate inhibitory activity of the expressed fusion product, PDEαβ was augmented with excess of GK-PDEγ, K-PDEγ, and the GST component without γ as a control, and hydrolysis of cGMP was monitored with a pH electrode [14]. As shown in Fig. 3, GST has little effect on PDE activity (trace 1), when compared with a control (c). In contrast, both GK-PDEγ and K-PDEγ completely inhibit hydrolysis of cGMP (traces 2 and 3) consistent with the localization of the inhibitory domain of PDEγ at the C-terminus [8,21–23] which is unaffected by the addition of GK. Addition of equal amounts of trypsin (arrow) destroys GK-PDEγ and K-PDEγ and restores activity. Restoration of activity from GK-PDEγ/PDEαβ (trace 3) was significantly slower than from K-PDEγ/PDEαβ, possibly due to the size of GK-PDEγ (40 kDa) which is four times larger than K-PDEγ (10 kDa). Addition of more trypsin to PDEαβ/GK-PDEγ increases the rate of cGMP hydrolysis (not shown). The results show that both the fusion protein and K-PDEγ inhibit activated PDE in a fashion comparable to native PDEγ.

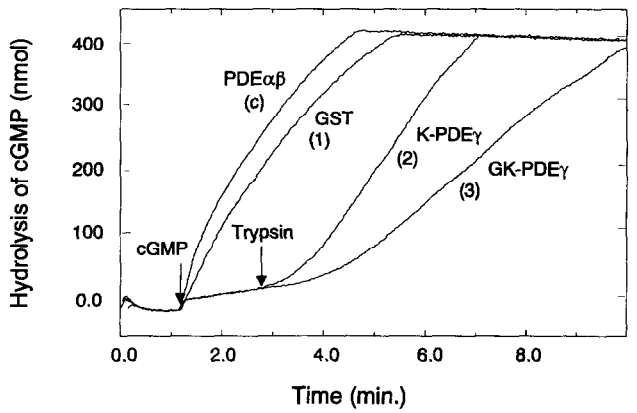

Fig. 3. Inhibition of cGMP PDE activity by GK-PDEγ and K-PDEγ. Trace (c), trypsin-activated bovine PDE as a control. The base-line was recorded for 90 s, then cGMP was added to a final concentration of 2 mM (at arrow marked 'cGMP') to start the cGMP hydrolysis. To trypsinized bovine PDE, purified GST (trace 1), K-PDEγ (trace 2), and GK-PDEγ (trace 3), were added. An aliquot of trypsin (at arrow marked 'trypsin') was added to traces 2 and 3 to reverse inhibition.

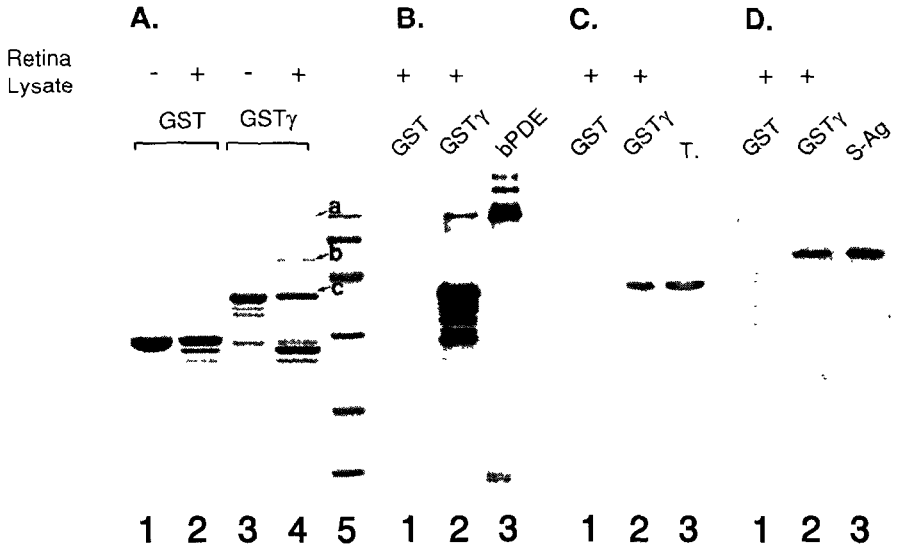

Fig. 4. Binding of retinal components to immobilized GK-PDEγ. A, binding of polypeptides present in retina lysates to glutathione beads containing GK-PDEγ. Lane 1, GST beads; lane 2, GST beads equilibrated with retina lysate; lane 3, GK-PDEγ-beads; lane 4, GK-PDEγ beads equilibrated with retina lysate Polypeptides bound are labeled a (large PDE subunits), β (arrestin), and c (transducin a subunit). Lane 5, molecular weight markers. B, Western blot of A2 (lane 1) and A4 (lane 2) probed with a PDE specific polyclonal antibody (MOE). Lane 3, bovine PDE large subunits as a control. C, Western blot of A2 (lane 1), and A4 (lane 2) probed with transducin a subunit antibody. Purified transducin is shown in lane 3. D, Western blot of A2 (lane 1) and A4 (lane 2) probed with arrestin specific antibody.

3.3. *Immobilized GK-PDEγ binds transducin a subunit charged with GTP, PDE large subunits and arrestin*

Apart from inhibition of activated PDE, the biological activity of PDEγ also consists of interaction with T_α^{GTP}. To demonstrate that the expressed fusion protein is biologically active, we exposed immobilized GK-PDEγ to bovine retinal extracts containing components soluble in hypotonic buffer containing 2 μM GTPγS (Fig. 4). A control experiment with GST (mobility 30 kDa) indicates binding of two unidentified proteins of a molecular weight lower than 30 kDa. Immobilized GK-PDEγ, but not the GST component, binds three retinal components termed a, b, c (Fig. 4A, lane 4) of the mobilities 40K, 50K, and 90K, respectively. The 90K component (a) was identified by its antigenicity to a polyclonal antibody (MOE, Fig. 4B) as the PDE large subunits (which do not separate in the gel system used in Fig. 4). Binding of the PDE large subunits is presumed to be due to the replacement of the endogenous γ subunit by immobilized GK-PDEγ [24]. The multiple antigenic bands in Fig 4B, lane 2 are presumably degradation products of GK-PDEγ which is exposed to various PMSF-insensitive proteases present in the hypotonic retinal extract. The 40K component (c) was identified as transducin a subunit in a Western blot with a T_α specific antibody (Fig. 4C). The transducin β subunit does not bind under these conditions, and the a subunit only binds to immobilized GK-PDEγ when eluted from ROS membranes in the presence of GTPγS (not shown). Thus, the expressed GK-PDEγ, by virtue of its inhibition of activated PDE and binding of its activator and the large subunits of PDE, is biologically active.

The 50K polypeptide (b) was identified as arrestin (48K protein, S-antigen) again by Western blotting of lane 4 (Fig. 4A) and probing with an anti-arrestin polyclonal antibody. The binding of arrestin to immobilized PDEγ was independent of ATP (1 mM), and tight enough to survive three stringent washings with isotonic buffer (see section 2). Purified T_α^{GTP} and PDE independently bind to immobilized PDEγ (not shown). The binding of purified arrestin, in the absence of transducin and PDE, to immobilized PDEγ is shown in Fig. 5. These results indicate that binding of the three individual components is independent from the presence of any of the other two.

3.4. *Putative role of arrestin*

The binding of arrestin, which is thought to quench PDE activation by binding to phosphorylated rhodopsin [18,25,26] thus inhibiting the generation of the PDE activator T_α^{GTP}, is surprising since no definitive role of arrestin downstream of rhodopsin in the phototransduction cascade has been established. It has been reported, however, that arrestin may have the ability to directly act on PDE, inhibiting its activation by T_α^{GTP} [27,28]. The term 'arrestin', in fact, was coined for 48K protein's proposed role as a deactivator of phosphodiesterase [27]. It is conceivable that arrestin may bind to PDE holoenzyme via its γ subunit, with possible points of contact also on the large PDE subunits, and that T_α^{GTP} may be unable to displace PDEγ and activate PDE. Although other laboratories have been unable to confirm a direct action of arrestin on PDE [18,26,29], our results clearly show that PDEγ can interact with

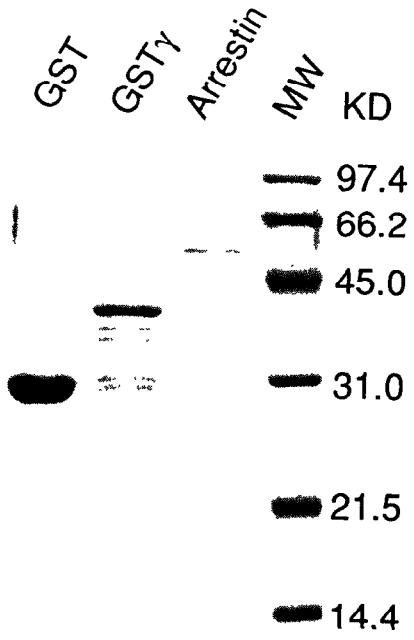

Fig. 5. Equilibration of GST and GK-PDEγ beads with purified arrestin. Glutathione beads charged with GST (lane 1), GK-PDEγ (lane 2) were incubated with purified arrestin (lane 3) as described in Fig. 4. Lane 5, molecular weight markers.

arrestin. A shared domain of sequence homology near the C-termini of T_α and arrestin [30] may participate in binding to sites on PDEγ [31,32]. A final analysis of a possible role of arrestin on modulation of PDE will have to await a more quantitative evaluation of the interaction of the components.

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REFERENCES

[1] Baehr, W., Devlin, M.J. and Applebury, M L. (1979) J. Biol. Chem. 254, 11669–11677.
[2] Hurley, J B. and Stryer, L. (1982) J Biol. Chem. 257, 11094–11099.
[3] Ovchinnikov, Y A., Lipkin, V.M., Kumarev, V.P., Gubanov, V V., Khramtsov, N V., Akhmedov, N.B., Zagranichny, V E. and Muradov, K G. (1986) FEBS Lett. 204, 288–292.
[4] Tuteja, N and Farber, D.B. (1988) FEBS Lett. 232, 182–186.
[5] Tuteja, N., Danciger, M., Klisak, I., Tuteja, R., Inana, G., Mohandas, T., Sparkes, R.S. and Farber, D.B. (1990) Genc 88, 227–232.
[6] Griswold-Prenner, I., Tuteja, N., Farber, D.B. and Fung, B.K.-K. (1989) Biochemistry 28, 6145–6150.
[7] Brown, R.L. and Stryer, L (1989) Proc. Natl Acad. Sci. USA 86, 4922–4926.
[8] Brown, R L. (1992) Biochemistry 31, 5918–5925.
[9] Qin, N., Pittler, S J. and Baehr, W (1992) J. Biol. Chem. 267, 8458–8463.
[10] Qin, N and Baehr, W. (1992) Invest. Ophthalmol Vis. Sci. 33, 1005.
[11] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.
[12] Kaelin Jr., W.G., Pallas, D.C., DeCaprio, J.A., Kaye, F.J. and Livingston, D.M. (1991) Cell 64, 521–532.
[13] Bradford, M.M. (1976) Anal. Biochem 72, 248–254.
[14] Liebman, P.A. and Evanczuk, T. (1982) Methods Enzymol. 81, 532–542.
[15] Wensel, T.G and Stryer, L. (1986) Prot. Struct Funct Genet. 1, 90–99.
[16] Baehr, W., Morita, E., Swanson, R and Applebury, M.L. (1982) J. Biol. Chem. 257, 6452–6460.
[17] Kuhn, H., Hall, S.W and Wilden, U. (1984) FEBS Lett. 176, 473–478.
[18] Wilden, U., Hall, S.W. and Kuhn, H (1986) Proc. Natl Acad. Sci. USA 83, 1174–1178
[19] Fung, B.K.-K., Hurley, J G. and Stryer, L. (1981) Proc. Natl. Acad. Sci. USA 78, 152–156.
[20] Clerc, A. and Bennett, N (1992) J. Biol Chem. 267, 6620–6627.
[21] Lipkin, V.M , Dumler, I L., Muradov, K.G., Artemyev, N O. and Etingof, R.N. (1988) FEBS Lett. 234, 287–290
[22] Takemoto, D.J , Hurt, D., Oppert, B. and Cunnick, J. (1992) Biochem. J 281, 637–643
[23] Lipkin, V M., Udovichenko, I.P., Bondarenko, V.A., Yurovskaya, A.A., Telnykh, E.V. and Skiba, N.B. (1990) Biomed. Sci. 1, 305–313.
[24] Wensel, T.G. and Stryer, L. (1990) Biochemistry 29, 2155–2161
[25] Thirkill, C.E., Tait, R.C., Tyler, N.K., Roth, A.M , and Keltner, J.L. (1992) Invest. Ophthalmol Vis Sci. 33, 2768–2772.
[26] Palczewski, K , Rispoli, G. and Detwiler, P B. (1992) Neuron 8, 117–126.
[27] Zuckerman, R. and Cheasty, J.E (1986) FEBS Lett. 207, 35–41.
[28] Zuckerman, R. and Cheasty, J.E. (1988) FEBS Lett 238, 379–384.
[29] Palczewski, K., Pulvermüller, A., Buczylko, J. and Hofmann, K.P. (1991) J. Biol. Chem. 266, 18649–18654.
[30] Yamaki, K., Tsuda, M and Shinohara, T (1988) FEBS Lett. 234, 39–43.
[31] Artemyev, N.O., Rarick, H.M., Mills, J.S., Skiba, N P. and Hamm, H E (1992) J. Biol. Chem. 267, 25067–25072.
[32] Rarick, H.M., Artemyev, N.O. and Hamm, H.E. (1992) Science 256, 1031–1033