Cyclic GMP phosphodiesterase from cattle retina

Amino acid sequence of the γ-subunit and nucleotide sequence of the corresponding cDNA

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The primary structure of the γ-subunit of cyclic GMP phosphodiesterase was determined by parallel analysis of the amino acid sequence of the protein and nucleotide sequence of the corresponding cDNA. The enzyme γ-subunit contains 87 amino acid residues, its N-terminal amino group being acetylated.

cyclic GMP phosphodiesterase *Amino acid sequence* *cDNA cloning* *Nucleotide sequence*

1. INTRODUCTION

To understand the mechanisms of transduction and amplification of the visual signal detailed information is necessary on the structure of proteins involved in these processes – rhodopsin, transducin and cyclic GMP phosphodiesterase. We utilized protein chemistry techniques to study the rhodopsin and transducin primary structures [1–4]. The same technique is hardly applicable in the case of α- and β-subunits of cyclic GMP phosphodiesterase because of its low content in the cell and rather long polypeptide chains. The enzyme from cattle retina consists of three subunits α, β, and γ of molecular masses 88, 84 and 11 kDa, respectively [5]. The γ-subunit is an internal inhibitor of the protein enzymatic activity, this inhibitor effect abolishes at the interaction of cyclic GMP phosphodiesterase and the GTP-bound form of transducin [6,7].

This study, a part of investigations into the primary structure and mechanism of function of the proteins transforming the visual signal, concerns the determination of the amino acid sequence of the cyclic GMP phosphodiesterase γ-subunit.

Along with direct protein sequencing, we undertook isolation of cDNA corresponding to the γ-subunit and established its nucleotide sequence. The complete coincidence of these structures testifies to the lack of post-translation processing of the protein and provides accurate results. (Preliminary results were published in [8,9].)

2. MATERIALS AND METHODS

Cyclic GMP phosphodiesterase was isolated as in [5]. The γ-subunit was separated from α- and β-subunits by reverse-phase HPLC (Synchropak C4, acetonitrile gradient in 0.1% trifluoroacetic acid) and eluted by 45% acetonitrile, and α- and β-subunits by 56%.

The γ-subunit was carboxymethylated, hydrolysed with trypsin and *Staphylococcus aureus* protease, and cleaved with cyanogen

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bromide and BNPS-skatole according to the conventional procedure [3,4,10].

The mixtures of the formed peptide fragments were separated by reverse-phase HPLC on Ultrasphere ODS or Synchropak C 18 columns (acetonitrile gradient in 0.1% trifluoroacetic acid). The sequence of the peptides was determined by Edman degradation (the amino acids being identified as their 1-dimethylaminonaphthalene-5-sulphonyl or phenylhydantoin derivatives) and also by automated degradation on a liquid-phase sequenator [3,4,10].

The poly(A^+)-fraction of RNA from cattle retina was obtained according to [11]. The first and second chains of cDNA were synthesized as described in [12]. The cohesive ends of the obtained cDNA were cleaved with nuclease S1 and the Klenow fragment. cDNA was then ligated with vector pUC 8 preliminarily cleaved with restrictase *Sma*I and diphosphorylated. *E. coli* cells, strain JM-101 were transformed with the produced recombinant plasmids as in [13]. As a result the cDNA clone library containing 5×10^5 recombinants was produced.

Nucleotide probes were synthesized by a phosphitamide method on a Gene 1M automated sequenator [14]. The nucleotide sequence of DNA fragments was detected by the modified [15] Maxam-Gilbert [16] procedure. The strategy for determination of the nucleotide sequence was the same as we used in [17]. The fragment was cleaved with restrictases *Sau*3AI, *Hin*fI, *Hpa*II, *Ava*II, and *Alu*I. After insertion of the 5′- or 3′-end radioactive label the chains of electrophoretically isolated subfragments were separated and their structures were analysed.

3. RESULTS AND DISCUSSION

Analysis of N-terminal amino acid residues of cyclic GMP phosphodiesterase subunits showed that their N-terminal amino groups were blocked. To determine the amino acid sequence of the γ-subunit we employed trypsin and *St. aureus* protease hydrolyses and cyanogen bromide cleavage. In the latter case the γ-subunit carboxymethylated with iodo[^{14}C]acetic acid was cleaved.

Fig.1 shows the reconstruction of the polypeptide chain of the enzyme γ-subunit. The N-terminal amino group of peptide SP-1 (1–9) was blocked, however, after its cyanogen bromide cleavage, the amino acid sequence (2–9) was elucidated. Mass spectrum of peptide SP-1 was recorded on an LC-MS 3303 mass spectrometer with 'ERIAD' ion source [18]; a molecular ion $(M+H)^+$ with *m/e* 1112 was observed. Determination of the peptide molecular mass revealed that the N-terminal methionine residue was acetylated. Additional hydrolysis with thermolysin was applied to study the structure of 49-meric peptide SP-2 (10–58). Six peptides were isolated from the hydrolysate by reverse-phase HPLC (Ultrasphere ODS).

To define the amino acid sequence of the central part of the molecule more precisely, the γ-subunit was hydrolysed by trypsin at arginine residues after modification of ϵ-amino groups of lysine residues with succinic anhydride. We isolated the 51-meric C-terminal peptide (37–87) from the hydrolysate by HPLC on a TSK-2,000 SW column. Sequencing of this peptide allowed detection of 37–60 amino acid residues in the γ-subunit and the overlapping between cyanogen bromide peptides Br-2 (18–57) and Br-3 (58–87).

Cleavage by BNPS-skatole was applied to localise the tryptophan residue in the γ-subunit polypeptide chain. The 71–75 sequence was determined by Edman degradation of the cleavage products; so we found the tryptophan residue to be in position 70. The amino acid sequence of peptide SP-6 (81–87) coincided with the C-terminal sequence of the γ-subunit (-Gly-Ile-Ile) established by carboxypeptidase A hydrolysis of the protein.

There were synthesized two unique complemen-

I. 5′-ATGAACCTGGAGCCCCCCAAGGCCGAGATCCGTTCCGCCACCCGCGTG-3′
Met Asn Leu Glu Pro Pro Lys Ala Glu Ile Arg Ser Ala Thr Arg Val

II. 3′-AGGCGGTGGGCGCACTACCCCCCCGGCCACTGGGGGGCATTCCCCGGG-5′
Ser Ala Thr Arg Val Met Gly Gly Pro Val Thr Pro Arg Lys Gly Pro

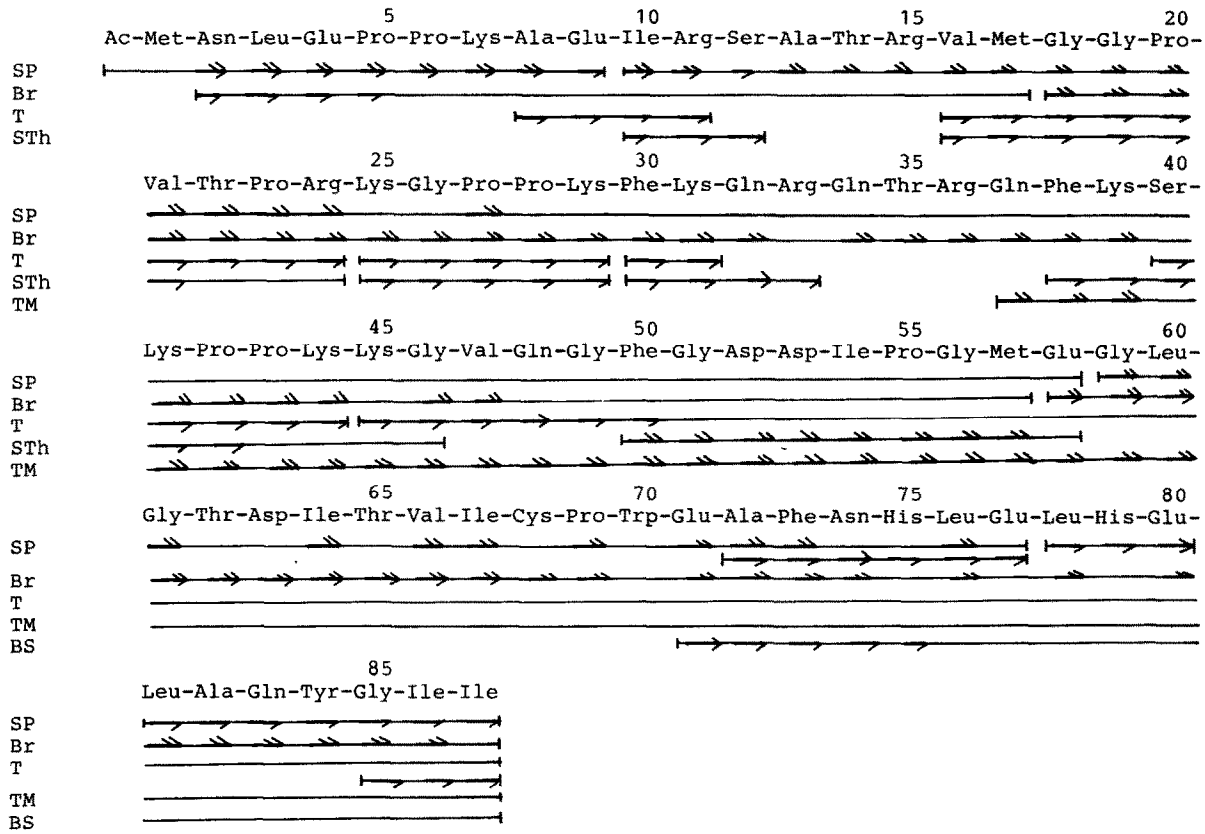

Fig.1. Amino acid sequence of the γ-subunit of cyclic GMP phosphodiesterase. The peptides obtained at protein cleavage with *S. aureus* protease (SP), cyanogen bromide (Br), trypsin (T), trypsin after modification of ϵ-amino groups of lysine residues (TM), BNPS-skatole (BS) as well as at hydrolysis of peptide SP-2 with thermolysine (STh), are shown. Amino acid sequences of peptides were analysed by Edman degradation with identification of DNS-derivatives (—►) and phenylhydantoins (—►) or by automated degradation on a sequenator (——).

tary overlapping nucleotide probes of 48 bp each by use of the amino acid sequence of the γ-subunit peptides:

To deduce the probe nucleotide sequences we took into account the codon usage in structural genes for rhodopsin and transducin [19–22]. Probe I was utilized for the primary screening of the clone library. Three clones pγ12, pγ20, pγ43 with positive hybridization signals were identified. The same clones were hybridized though weaker, with probe II. Detection of the sequence of the γ-subunit cDNA revealed that the structure of probe I differs from the true sequence in 5 positions and the probe II structure in 10 positions; it evidently determines the difference in intensity of hybridization signals.

Restriction analysis of the cloned DNA fragments isolated from the three obtained clones showed their close homology. Clone pγ12 was chosen for the analysis of the cDNA nucleotide sequence.

The complete nucleotide sequence of the studied cDNA fragment containing 833 bp is outlined in fig.2. Comparison of the deduced amino acid sequence of the protein and the amino acid sequence of the γ-subunit peptides designated that the isolated cDNA fragment contained the whole structural moiety of the gene for this protein of 261 bp and 5′- and 3′-untranslated regions (54 and 518 bp, respectively). Termination codon TAG is in position 262–264, and 3′-untranslated region in position 756–761 contains the signal of

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      -54                                                    -1
       GCGTGAGGGAGTCCAGAAGCTGAAGGTCACTGCGGGATCTCTGCCAACCTGGCC

  ATG AAC CTG GAG CCA CCC AAG GCC GAG ATC CGG TCG GCC ACC AGG     1-45
AcMet-Asn-Leu-Glu-Pro-Pro-Lys-Ala-Glu-Ile-Arg-Ser-Ala-Thr-Arg-   1-15

  GTG ATG GGG GGA CCC GTC ACT CCC AGG AAA GGG CCC CCG AAA TTT    46-90
  Val-Met-Gly-Gly-Pro-Val-Thr-Pro-Arg-Lys-Gly-Pro-Pro-Lys-Phe-   16-30

  AAG CAG CGG CAA ACC AGG CAG TTC AAG AGC AAG CCC CCC AAG AAA    91-135
  Lys-Gln-Arg-Gln-Thr-Arg-Gln-Phe-Lys-Ser-Lys-Pro-Pro-Lys-Lys-   31-45

  GGT GTC CAA GGG TTT GGT GAT GAC ATC CCT GGA ATG GAA GGC CTG   136-180
  Gly-Val-Gln-Gly-Phe-Gly-Asp-Asp-Ile-Pro-Gly-Met-Glu-Gly-Leu-   46-60

  GGA ACA GAC ATC ACC GTC ATC TGC CCG TGG GAG GCC TTC AAC CAC   181-225
  Gly-Thr-Asp-Ile-Thr-Val-Ile-Cys-Pro-Trp-Glu-Ala-Phe-Asn-His-   61-75

  CTG GAG CTG CAC GAG CTG GCC CAG TAC GGC ATC ATC TAG CCCTGGA   226-271
  Leu-Glu-Leu-His-Glu-Leu-Ala-Gln-Tyr-Gly-Ile-Ile TER           76-87

  CCCCCGCCCTCAGCCCCTCTACTCCGCTGCCCACCCTGACCCCCTGCTCAAGATTCCTG   272-330

  TGAGGAGAGCTGTGCCCCGGGAGGTCCAGAGTGTCTGGATTGTGTCTGGAGACCCTCAC   331-389

  AGGGCGGCAGCCTGGAGCCTCCTGAATGCTAGTTACCAGGAGCCCACCAGTTCCCTTCA   390-448

  GGACACCCTCTCGGGGCAGCCAGGCTCTGCTTAACTTCCAGAAACACTGGTCCACAGAC   449-507

  CCTCTCCTCCCCAGGCTGGAAAGCTAGGGCAGGCCTCCCAGTGGTGTCTGCCACACCCC   508-566

  GCCTCCTGGCCTGACTGTCTGGGGGTGAGAACGGGCTCCCCTCACTAGCCTTTCCCAGT   567-625

  TGAAGCCGTTGGGCCAGCAGGTGGATGCCAGGAGTCCTGCAGGCGTCAGACAATGAGAA   626-684

  CCCCCTGGACCAGTCACACCAGTAGGAAGCTTGTCCTTTCCAACGTGGCCCATGCTCGC   685-743

  TGTCCTGTTTCAAATAAAGTTAGCCGTGCTCCCCAA                          744-779
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Fig.2. Nucleotide sequence of cDNA of the cyclic GMP phosphodiesterase γ-subunit from bovine retina and the corresponding amino acid sequence of the protein.

polyadenylation – AATAAA.

Parallel analysis of the protein amino acid sequence and the nucleotide sequence of the corresponding cDNA provided the accurate results. In particular, detection of the cDNA structure confirmed the positions 35 and 63 for threonine and aspartic acid residues in the γ-subunit, that was not quite clear from the study of the protein amino acid sequence. On the other hand, amino acid sequences of the peptides were useful in finding nucleotide sequences of certain cDNA fragments.

The γ-subunit polypeptide chain consists of 87 amino acid residues that corresponds to the molecular mass 9700 Da; its amino acid composition: Asp 3, Asn 2, Thr 5, Ser 2, Glu 6, Gln 5, Pro 10, Gly 10, Ala 4, Cys 1, Val 4, Met 3, Ile 6, Leu 5, Tyr 1, Phe 4, His 2, Lys 8, Arg 5, Trp 1. The N-terminal amino group of the protein is acetylated. The γ-subunit is the basic protein – 9 residues of dicarbonic amino acids per 13 residues

of basic amino acids. It is noteworthy that 10 of the basic residues are concentrated in the comparatively short region of the polypeptide chain (24–45) having no residue of dicarbonic amino acid. This region of the γ-subunit is assumed to be functionally important, because the inhibitory action of the γ-subunit on the phosphodiesterase activity of the enzyme decreases rapidly when treated with trypsin in a low concentration [6,7].

At present the cDNA clone library is analysed with nucleotide probes synthesized on the basis of amino acid sequences of the peptides of the enzyme α- and β-subunits.

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