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The primary structure of the 70 kDa subunit of bovine soluble guanylate cyclase

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The primary structure of the 70 kDa subunit of soluble bovine guanylate cyclase, which catalyzes the formation of cyclic GMP from GTP, has been determined. The alignment of six different clones out of two bovine libraries yielded a total of 3.1 kb with a coding region of 1857 bases. The open reading frame encodes a protein of 619 amino acids and a molecular mass of 70.5 kDa. Antibodies raised against a synthetic peptide, which corresponded to the C-terminus of the deduced sequence precipitated guanylate cyclase activity from guanylate cyclase-enriched preparations.

cDNA sequence; Peptide antibody; Guanylate cyclase

1. INTRODUCTION

In most tissues, at least two guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) (GC) isoenzymes exist, a soluble and a membrane-bound form. The enzymes differ in biochemical and antigenic properties [1,2]. cGMP acts as an intracellular messenger activating cGMP-dependent protein kinases [3] and regulating cGMP-sensitive ion channels [4]. Although the role of cGMP is in general not well understood, cGMP is an established mediator at least in vascular smooth muscle relaxation and retinal phototransduction [1].

Several laboratories have purified soluble GC from different tissues to apparent homogeneity [1]. The purified enzyme has a native apparent molecular mass of 150 kDa and consists of two subunits, possibly a catalytic and a regulatory one with molecular masses between 70 and 82 kDa on SDS gels. Here we report on cDNA cloning and sequencing of the 70 kDa subunit of soluble GC. Data obtained with a precipitating antibody against a synthetic C-terminal peptide verify the identity of the cDNA clones.

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession no. Y00770

2. MATERIALS AND METHODS

Soluble GC was purified from bovine lung as in [5]. The smaller (70 kDa) subunit (70 μg) was isolated by electroelution out of Coomassie blue-stained gels [6]. One-third of the eluted protein was directly analyzed by N-terminal sequencing in a gas-phase sequencer [7]. The rest of the eluted protein was digested with 6 μg TPCK trypsin (Worthington, Redford, USA). Tryptic peptides were separated by HPLC on a Bakerbond wide pore C4 column (Baker, Groß-Gerau, FRG), using 0.1% trifluoroacetic acid and a linear gradient of 0–60% acetonitrile (120 min). Individual peaks were collected and analyzed by N-terminal sequencing.

The cDNA library from bovine lung was constructed in pEX [8]; a cDNA library from bovine pituitary (kindly provided by Dr P. Seeburg, Heidelberg) was constructed in λgt11 [9]. The procedures for screening were as in [10]. The oligonucleotide probe

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   T  T  G  C  G  C  T  C
ATCAACGTCTCAGAATATACATAT
         T        C
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labelled with ^{32}P at the 5′-end was used for hybridization at 37°C. Washing was carried out at 37°C in 6 × SSC (1 × SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0), 0.1% SDS. The inserts of clones A and N were radiolabelled by random oligopriming and extension of double-stranded DNA [11]. DNA was sequenced according to the dideoxy chain-termination method [12,13].

Peptide synthesis was as in [14]. The synthetic peptide, CSRKNTGTEETEQDEN, was purified by reversed-phase HPLC and coupled through an N-terminal cysteine as in [15]. Polyclonal antibodies were raised in New Zealand rabbits. Soluble GC-enriched preparations (5–15 μg protein) were incubated with 10 μl preimmune serum or antiserum 70/3 in 50 mM Tris-HCl (pH 8.0), containing 2 mM EDTA and 2 mM DTT at 4°C overnight. Protein A-Sepharose (5 mg) was added (1 h), the supernatant removed, and the pellet washed and resuspended. Elution was carried out for 8 h at 4°C with 10 μg peptide. GC activity was determined before and after immunoprecipitation and in the eluate. Immunoblot analysis was performed as in [16].

3. RESULTS AND DISCUSSION

The N-terminal peptide (I: MYGFVN) and seven internal peptides (II, INVSEYTY; III, SVLPPSVANEL; IV, EAQLD; V, GLYSDI; VI, EFLQNLDALHDHLA; VII, IIYDD; VIII, SVLPP) of the isolated 70 kDa subunit of soluble GC were determined by sequence analysis with a gas-phase sequencer, providing partial sequence information throughout the protein.

A cDNA library derived from bovine lung poly(A)$^+$ RNA with approx. 200000 clones was screened by hybridization with radiolabelled oligonucleotides representing back-translation of the amino acid sequence of peptide II (INVSEYTY). One positive clone, designated A, with an insert of 2364 bp was isolated; the insert was subcloned and sequenced (fig.1). The first 489 bp of the 5′-region contained stop codons in all reading frames and a consensus intron/exon boundary sequence. This region was followed by 681 bp with an open reading frame. In the deduced protein sequence, an 8 amino acid segment (bases 998–1021) was identical with peptide II, of which the sequence of the oligonucleotide probe was

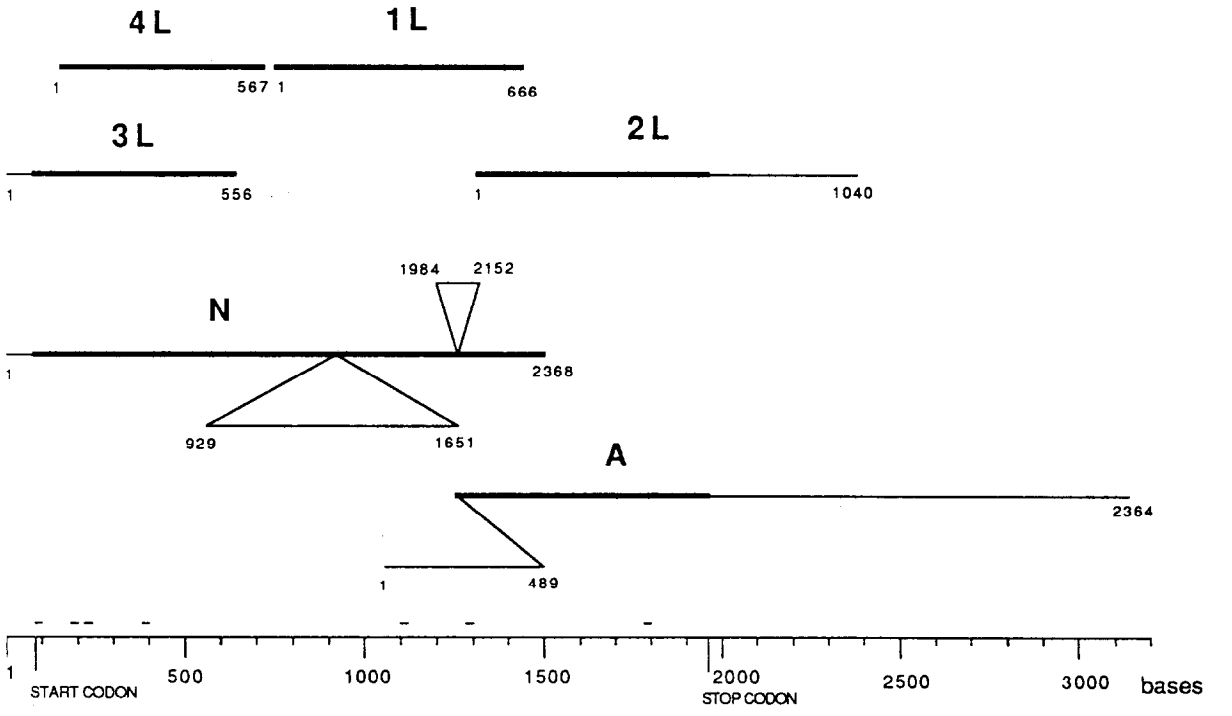

Fig.1. Diagram illustrating the alignment of the six overlapping cDNA clones. Clones were derived from a bovine pituitary cDNA library (1L–4L) and a bovine lung cDNA library (N, A). The lengths of the individual clones (1L, 2L, 3L, 4L, N, A) are given below. The amino acid coding region in each cDNA clone is shown as a bold line. The numbering of the composite cDNA sequence is shown as a ruler at the bottom of the diagram. Dashes (–) above the ruler mark the position of the peptides obtained from protein sequencing. The introns in clones A and N are shown by triangles.

derived. Within the same reading frame, the sequence of peptide III was identified (500–533). At the 3′-end, clone A contained 1191 bp of an untranslated region which included the polyadenylation signal AAUAAA. Clone A did not cover the N-terminal part of the coding region. Therefore, the insert of clone A was used to rescreen the library. This yielded clone N, containing an insert of 2368 bp, which was subcloned and sequenced. The 3′-end (214 bp) of clone N was identical with the bases 490–704 of clone A, which were shown to be part of the coding sequence. In the translated sequence of clone N, 6 peptides (III–VIII) and the N-terminal 6 amino acids of the 70 kDa protein were identified. However, the open reading frame of clone N containing the sequences corresponding to the peptides was interrupted by two regions (929–1651; 1984–2152) with stop codons in all frames. These regions were, with one exception, flanked by typical exon/intron and intron/exon boundaries, indicating the presence of introns or partially spliced introns in both clones. This may be due to the fact that total RNA (including nuclear components) was used to construct the cDNA library. No completely spliced clones were obtained from the bovine lung library. Therefore, a second cDNA library derived from bovine pituitary poly(A)$^+$ RNA was screened, using clone N as hybridization probe. Four positive clones (1L, 2L, 3L, 4L) were isolated, and the inserts of 666, 1040, 556, 567 bp were subcloned and sequenced. These clones were partially overlapping and covered an open reading frame of length 1858 bp. A gap of 50 bases between the 3′-end of clone 4L and the 5′-end of clone 1L was overlapped by the open reading frame in clone N. Sections 929–1651 and 1984–2152 of clone N and 1–489 of clone A (fig.2), which contained stop codons in all reading

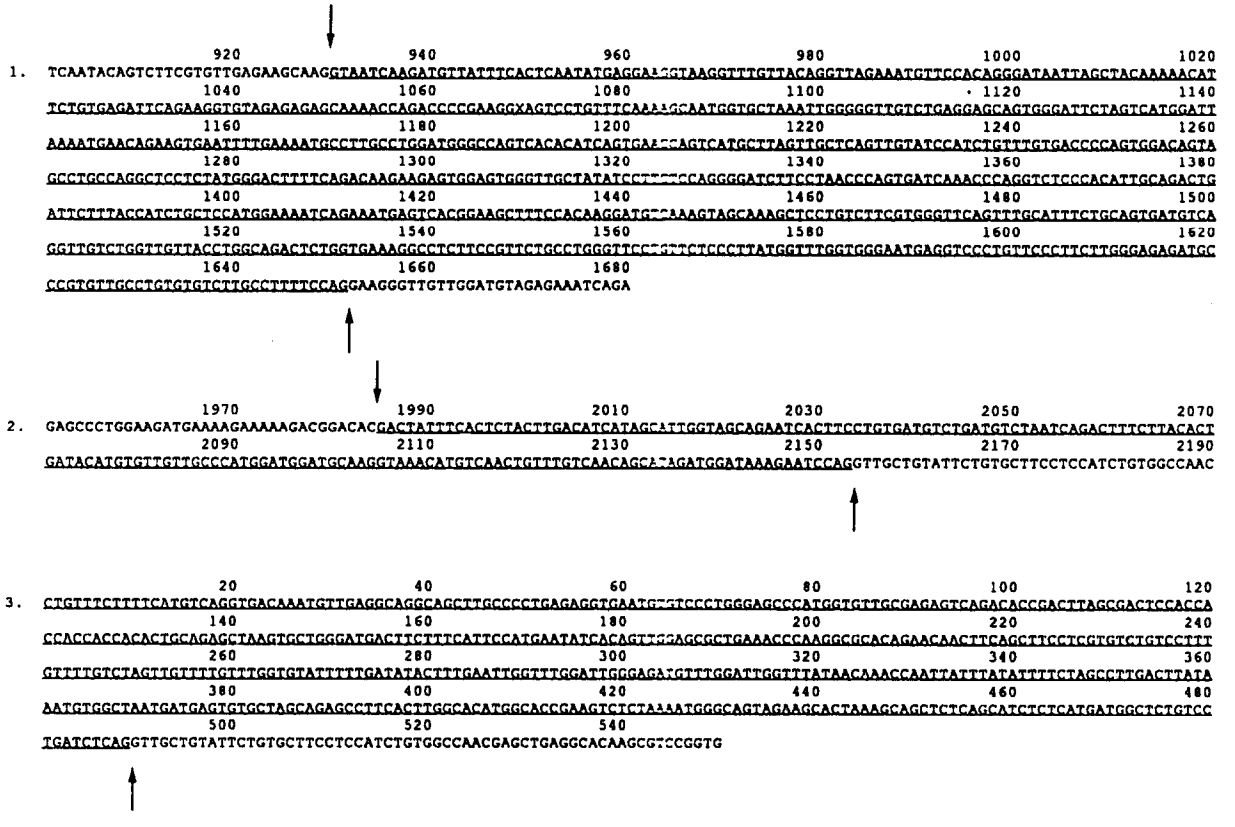

Fig.2. Intron sequences of clones N and A. Clones N and A were derived from a bovine cDNA library. The sequences of the introns are underlined. The exon/intron boundaries are marked by arrows. (1) Bases 929–1651 of clone N; (2) bases 1984–2152 of clone N, (3) bases 1–489 of clone A.

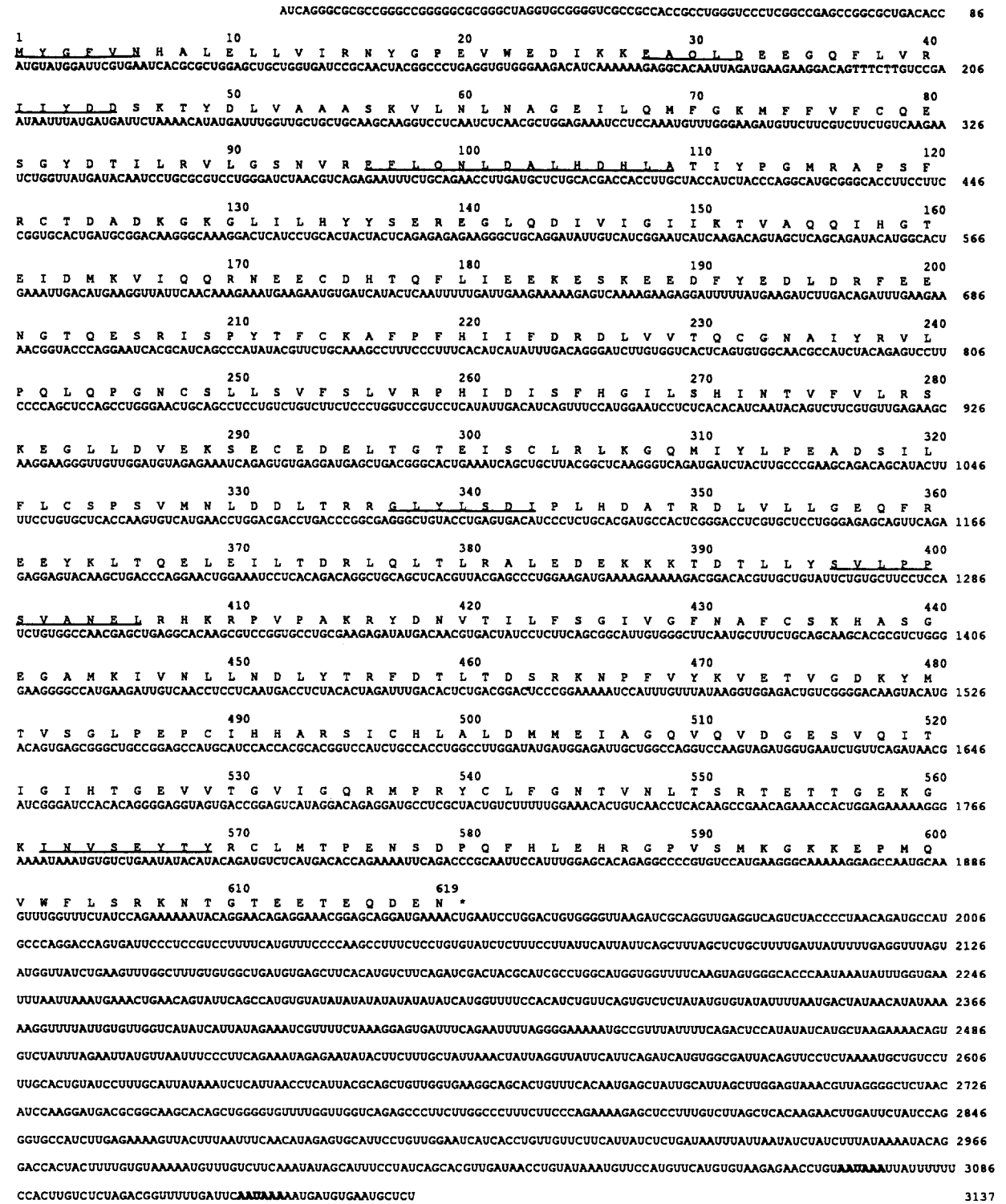

Fig.3. Complete nucleotide and deduced amino acid sequences of the 70 kDa subunit of soluble GC. The peptide sequences obtained from protein sequencing are underlined. Possible polyadenylation signals (AAUAAA) are printed in bold letters.

frames and the intron/exon boundaries, were not present in clone 1L.

Fig.3 shows the complete nucleotide sequence derived from clones 1L, 2L, 3L, 4L, A and N as illustrated in fig.1. The sequence is 3137 bp long, contains an 86 bp untranslated region at its 5′-end, a coding region of 1857 bp and a 1191 bp non-coding region at its 3′-end. The non-coding 3′-region contains a polyadenylation signal AAUAAA but lacks a poly(A) tail. The open reading frame codes for a protein of 619 amino acids with a calculated molecular mass of 70.503 kDa, which corresponds well with the apparent value for the 70 kDa subunit of soluble GC. The sequences of all tryptic peptides and of the N-terminal peptide occur within the open reading frame. Blot hybridization analysis of bovine brain poly(A)$^+$ RNA with a 70 kDa subunit of GC cDNA probe exhibited a hybridizable RNA with an estimated size of 3.2 kb (fig.4).

To confirm that the deduced amino acid sequence was indeed the 70 kDa subunit of GC, a peptide (CSRKNTGTEETEQDEN) was synthesized according to the C-terminal sequence of the deduced amino acid sequence. Antiserum raised in rabbits against this peptide precipitated more than 70% of GC activity from different soluble GC-enriched preparations. Rabbit preimmune serum did not precipitate GC activity. The immunoprecipitated GC activity was quantitatively eluted by the peptide used as antigen. The immunoblot analysis of the eluted immunoprecipitate showed a single band at about 70 kDa (not shown).

A comparison of the protein sequence of the 70 kDa subunit of soluble GC with the protein sequence data library of the National Biomedical Research Foundation (NBRF) and of a translation of the European Molecular Biology Laboratory (EMBL) nucleotide sequence database revealed no significant homologies. A hydropathy plot did not show any large hydrophobic or hydrophilic regions. As GC catalyzes the conversion of GTP to cGMP, we searched for possible GTP-binding sites. Three motifs (GxxxxGKS, NkxD, DxxG) are highly conserved between different GTP-binding proteins including the elongation factors EF-Tu and EF-1α G-protein α-subunits and ras proteins [17]. These motifs are not present in the deduced amino acid sequence of the 70 kDa subunit of GC.

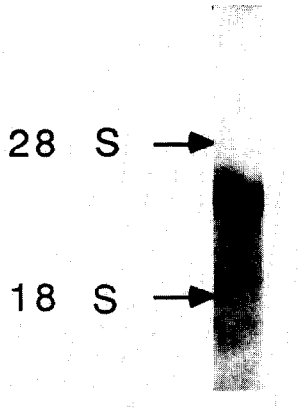

Fig.4. Autoradiogram of blot hybridization analysis of bovine brain poly(A)$^+$ RNA with a 70 kDa subunit of GC cDNA probe. Total RNA was isolated as in [21]. Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose column chromatography. 3 μg poly(A)$^+$ RNA were separated on formaldehyde gels and blotted on nitrocellulose filters [10]. The hybridization probe was a fragment excised from clone A (bases 879–2364) containing the C-terminal part of the coding region and the non-coding region. Labelling of the probe and hybridization are described in section 2. Positions of the 18 S and 28 S rRNAs are indicated.

The fact that no known GTP-binding regions could be identified within the sequence of the 70 kDa subunit presented here does not necessarily mean that the cloned protein represents the regulatory but not the catalytic subunit of GC. The enzyme catalyses the conversion of GTP to cGMP with pyrophosphate as a side product but does not hydrolyze GTP to GDP as do the GTP-binding proteins mentioned above. Therefore, the regions involved in GTP-binding and conversion by GC and by G-proteins should not necessarily be related. The only nucleotide triphosphate pyrophosphate lyase (cyclizing) enzymes sequenced so far are bacterial and yeast adenylate cyclases [18–20], which do not show significant homologies to the present protein sequence of GC.

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Note added in proof

After submission of this paper, the primary structure of the spermatozoan membrane GC of sea urchin *Arbacia punctulata* was published [(1988) Nature 334, 708–712]). There is strong similarity in a stretch of 48 amino acids between the 70 kDa subunit of the bovine soluble GC and a C-terminal region of the receptor-linked, membrane-bound GC, where 23 identical residues can be observed:

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383                                                  430
LEDEKKKTDTLLYSVLPPSVANELRHKRPVPAKRYDNVTILFSGIVGF soluble GC
|  || ||  ||   |||| |  |      |      | | | || ||||
LQKEKAKTEQLLHRMLPPSIASQLIKGISVLPETFDMVSIFFSDIVGF membrane GC
852                                                  899
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Since both enzymes catalyze the same reaction but are regulated differently, it is highly likely that the 70 kDa subunit represents the catalytic subunit of the bovine soluble GC.