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Primary structure of the 5 S subunit of transcarboxylase as deduced from the genomic DNA sequence

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Transcarboxylase from *Propionibacterium shermanii* is a complex biotin-containing enzyme composed of 30 polypeptides of three different types. It is composed of six dimeric outer subunits associated with a central cylindrical hexameric subunit through 12 biotinyl subunits; three outer subunits on each face of the central hexamer. Each outer dimer is termed a 5 S subunit which associates with two biotinyl subunits. The enzyme catalyzes a two-step reaction in which methylmalonyl-CoA and pyruvate form propionyl-CoA and oxalacetate, the 5 S subunit specifically catalyzing one of these reactions. We report here the cloning, sequencing and expression of the monomer of the 5 S subunit. The gene was identified by matching amino acid sequences derived from isolated authentic 5 S peptides with the deduced sequence of an open reading frame present on a cloned *P. shermanii* genomic fragment known to contain the gene encoding the 1.3 S biotinyl subunit. The cloned 5 S gene encodes a protein of 519 amino acids, M_r 57,793. The deduced sequence shows regions of extensive homology with that of pyruvate carboxylase and oxalacetate decarboxylase, two enzymes which catalyze the same or reverse reaction. A fragment was subcloned into pUC19 in an orientation such that the 5 S open reading frame could be expressed from the lac promoter of the vector. Crude extracts prepared from these cells contained an immunoreactive band on Western blots which co-migrated with authentic 5 S and were fully active in catalyzing the 5 S partial reaction. We conclude that we have cloned, sequenced and expressed the monomer of the 5 S subunit and that the expressed product is catalytically active.

Transcarboxylase; Biotin; Pyruvate; Oxalacetate; Sequence; 5 S subunit

1. INTRODUCTION

The biotin-containing enzyme transcarboxylase (EC 2.1.3.1) (TC) from *Propionibacterium shermanii* is made up of three types of subunits, a central cylindrical hexameric 12 S subunit, six 5 S subunits and twelve 1.3 S biotinyl subunits [1,2]. In the intact enzyme, the 1.3 S biotinyl subunits link the six outer 5 S subunits, three on each face, to the central 12 S subunit and serve as the carboxyl carrier between the CoA ester sites on the 12 S subunit and the keto acid sites on the 5 S subunits (for reviews, see [3–5]). Like other biotin enzymes, the overall reaction catalyzed by transcarboxylase consists of two partial reactions, however, the reaction does not involve the fixation of CO_2 or a decarboxylation but rather a carboxyl transfer [6].

$$\begin{array}{l} \mathrm{COO^-} \\ \mathrm{|} \\ \mathrm{CH_3\text{-}CH\text{-}COSCoA} + 1.3\ \text{S-biotin} \overset{12\ \mathrm{S}}{\rightleftharpoons} \mathrm{CH_3CH_2COSCoA} \\ + 1.3\ \text{S-biotin-COO}^- \end{array} \quad (1)$$

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Abbreviations: TC, transcarboxylase; WT, wild type; MMCoA, methylmalonyl-CoA; Bct, biocytin.

$$\mathrm{CH_3COCOO^-} + 1.3\ \text{S-biotin-COO}^- \overset{5\ \mathrm{S}}{\rightleftharpoons} \mathrm{^-OOCCH_2COCOO^-} + 1.3\ \text{S-biotin} \quad (2)$$

$$\text{SUM:methylmalonylCoA} + \text{pyruvate} \rightleftharpoons \text{propionyl CoA} + \text{oxalacetate} \quad (3)$$

The 12 S subunit specifically catalyzes the transfer of the carboxyl of methylmalonyl CoA to the biotin of the 1.3 S subunit and the 5 S subunit catalyzes the transfer of the carboxyl from biotin to pyruvate [7].

The 5 S subunit is a metallo-protein containing Co^{2+} and Zn^{2+}. It is a homodimer of molecular weight 120,000 Da [8]. The subunit contains regions necessary for dimerization, interaction with the 1.3 S subunit and ketoacid binding. Fluorescence studies have demonstrated that a peptide consisting of residues 15–26 of the 1.3 S subunit binds to the 5 S subunit [9] and that at least one tryptophan residue is involved in the pyruvate binding site [10]. The binding of pyruvate has been shown to quench the tryptophan fluorescence of the 5 S subunit [10]. Chemical modification of tryptophan residues of the 5 S subunit by *N*-bromosuccinimide decreased the activity of TC complexes assembled from modified 5 S subunits [10].

We report here the cloning and sequencing of the 5 S gene from *Propionibacterium shermanii*, expression in *E. coli*, and demonstration of the activity of the recom-

binant protein. We are also able to identify one of the substrate binding site residue tryptophan as Trp-73.

2. MATERIALS AND METHODS

2.1. *Cloning and sequencing of the 5 S subunit*

A 7.1 kb fragment was isolated from a *P. shermanii* genomic library (pPS-Lib) using a 1.7 kb 1.3 S containing cDNA [11] as a probe. The library contained size fractionated 5–10 kb fragments generated by partial digestion with *Sau*3A and ligated into the *Bam*HI site of pUC19. The 7.1 kb fragment was extensively restriction-mapped and random fragments subcloned into M13mp9 for sequencing by the dideoxy chain termination method [12] using the large fragment (Klenow) of DNA polymerase I or Sequenase. The region of the 7.1 kb clone encoding the 5 S subunit was positioned by matching amino acid sequences derived from peptides isolated from trypsin treated transcarboxylase or isolated 5 S subunit. From the tryptic peptide sequences, 150 residues were matched to the deduced amino acid sequence of 5 S. The putative 5 S subunit encoding region was sequenced by subcloning restriction fragments from this area of the 7.1 kb clone into M13mp9. The sequence from overlapping restriction fragments was used to generate the entire sequence of the region.

2.2. *Expression of the 5 S subunit in* E. coli

The 5 S subunit was expressed from a 2.7 kb *Eco*RI fragment which encoded the entire 5 S gene and the N-terminal half of the 12 S gene subcloned into pTZ18. This subclone, called pLac5S, placed the 5 S gene under the control of the lac promoter and was used to transform *E. coli* strain HB101 yielding clones in which the 5 S protein was detected by Western blotting. Cultures of *E. coli* were grown in a medium containing yeast extract (10 g), tryptone (16 g), NaCl (10 g), $CoCl_2$ (0.1 mM), $ZnSO_4$ (0.1 mM), ampicillin (100 μg/ml) and IPTG (1 mM) per liter.

2.3. *Electrophoresis*

Protein samples were fractionated by 12.5% polyacrylamide gel electrophoresis according to Laemmli [13]. DNA sequence analysis was accomplished by 6% polyacrylamide gel electrophoresis in the presence of 7 M urea [12].

2.4. *Western blot analysis*

SDS-PAGE was performed as described above. After electrophoresis the gel was removed from the plate and then electrotransferred to a PVDF membrane in a buffer solution of 25 mM Tris-HCl, 192 mM glycine, pH 8.3, 10% methanol at 1.0 A for 1 h. After transfer, the PVDF membrane was washed with Tris-buffered saline (TBS) and blocked with 5% non-fat dry milk in TBS at room temperature for 1 h. The membrane was then washed with TBS containing Tween 20 (0.05%)(TTBS) two times, 5 min each time and then incubated with anti-5 S antibody for 4 h at room temperature. After the first antibody reaction, the membrane was washed with TTBS, then incubated with GAR-AP (goat anti-rabbit IgG, alkaline phosphatase linked from Calbiochem) for 1 h at room temperature. After this second antibody reaction, the membrane was washed with TTBS twice, TBS once and reacted with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to visualize the bands [14]. *E. coli* expressed 5 S is called 5 S wild type (WT).

2.5. *Synthesis of [4-^{14}C]oxalacetate*

To 0.12 units of carboxytransphosphorylase [15] that had been incubated for 15 min at room temperature in 40 μl of 0.05 M phosphate buffer plus 1 μl of 100 mM DTT (total volume, 50 μl) is added 3 μl of 1 M phosphate buffer (pH 6.6), 7 μl of 0.1 M $MgCl_2$, 10 μl of 0.3 M $KH^{14}CO_3$ (6,000 cpm/nmol) and 25 μl of 20 mM P-*enol*pyruvate. The formation of oxalacetate is determined spectrophotometrically with malate dehydrogenase using 5 μl aliquots. The reaction is usually complete in 20 min and is stopped by the addition of an equal volume of 0.1 N HCl. The oxalacetate content of the acidified solution is determined spectrophotometrically using malate dehydrogenase. The radioactivity is determined by adding 5 μl to 40 μl of 0.1 M phosphate, pH 6.6, and then 25 μl of Reducing Mix. (The Reducing Mix contains per 25 μl: 2.4 units of malate dehydrogenase; 2.4 units of lactate dehydrogenase; 20 μg of NADH and 1.5 mmol of $KHCO_3$.) After 5 min at room temperature, 50 μl of 1 N HCl is added and the solution is evaporated to dryness under vacuum at 45°C. The dried samples are dissolved in 200 μl of H_2O and, after 5 min (with occasional shaking), 2.8 ml of scintillant is added and the radioactivity determined. Usually about 0.3 μmol of [4-^{14}C]oxalacetate is obtained and it is used without further purification.

2.6. *Measurement of partial reaction with the 5 S subunit*

The partial reactions were conducted in a water bath at 25°C in 7 ml borosilicate glass (Fisher Scientific). The vials are placed in a tilted test tube rack so that the solution accumulated at one edge and the solutions were introduced at this edge using Hamilton syringes. The additions were made in the following order: 30 μl of 1.0 M phosphate buffer, pH 6.6, and H_2O so that the final volume will be 85 μl, 1.5 nmol of the 1.3 S WT subunit and 10 μl containing approximately 0.8 μg of the 5 S subunit in 0.5 M phosphate buffer, pH 6.6. The reaction was started by addition of 30 nmol of [4-^{14}C]oxalacetate contained in 62.5 mM phosphate buffer, pH 6.6. The vial was immediately shaken by hand and after 60 seconds, the reaction was stopped by adding 25 μl of Reducing Mix (The Reducing Mix contains for 25 μl: 2.4 units of malate dehydrogenase; 2.4 units of lactate dehydrogenase, 20 μg NADH and 1.5 mmol $KHCO_3$.) to reduce the oxalacetate to malate and after 5 min, 50 μl of 1 N HCl is added prior to drying at 45°C under vacuum. The dried samples were dissolved in 200 μl of H_2O and after 5 min (with occasional shaking), 2.8 ml of scintillant (Formula-963, NEN Research Products), is added and the radioactivity determined from the average of duplicate 10 min counts. The total nmol of carboxylated biotin associated with the complex was calculated by dividing the difference in acid stable counts, i.e. between the control (without enzyme) and the test (with enzyme), by the specific radioactivity of the [4-^{14}C]oxalacetate.

2.7. *Sequence determination*

N-terminal sequence analyses were carried out in an Applied Biosystems Model 470A sequencer [16]. Phenylthiohydantoins (PTH) were identified by complementary HPLC systems [17]. Quantitative evaluation of the PTH amino acids was as described by Smithies et al. [18] and Machleidt and Hofner [19].

2.8. *Amino acid analysis*

For amino acid analysis, the peptides were hydrolyzed in vacuo in 6 N HCl for 18 h at 110°C. The amino acid composition was then determined by a modified PTC method as described by Phillips and Wood [20].

2.9. *Tryptic digestion*

Approximately 20 nmol of 5 S subunit or transcarboxylase (in 300 mM potassium phosphate buffer, pH 6.5, containing 0.1 mM DTT and 0.1 mM PMSF was dialyzed against 100 mM NH_4HCO_3, pH 8.0, at 4°C for 48 h with three changes of buffer before trypsinization. TPCK-treated trypsin (Worthington) was added in a protein to trypsin ratio of 100:1 and incubated at 37°C for 24 h. The reaction was stopped by the addition of a 5-fold excess of a mixture of DFP and PMSF and the solution was lyophilized. The residue was dissolved in 0.1% (v/v) TFA and used for subsequent HPLC analysis.

2.10. *Reverse-phase HPLC analysis*

The tryptic digests of 5 S subunit were separated on a reverse phase C_4 column (Synchropak) using a DuPont HPLC system. Peptides were eluted with a linear gradient consisting of 0.1% TFA in H_2O (solvent A) and 0.1% TFA in CH_3CN (solvent B). The peptide elution was monitored at 220 nm. Selected peptides were subsequently repurifed on a C_{18} reverse phase column.

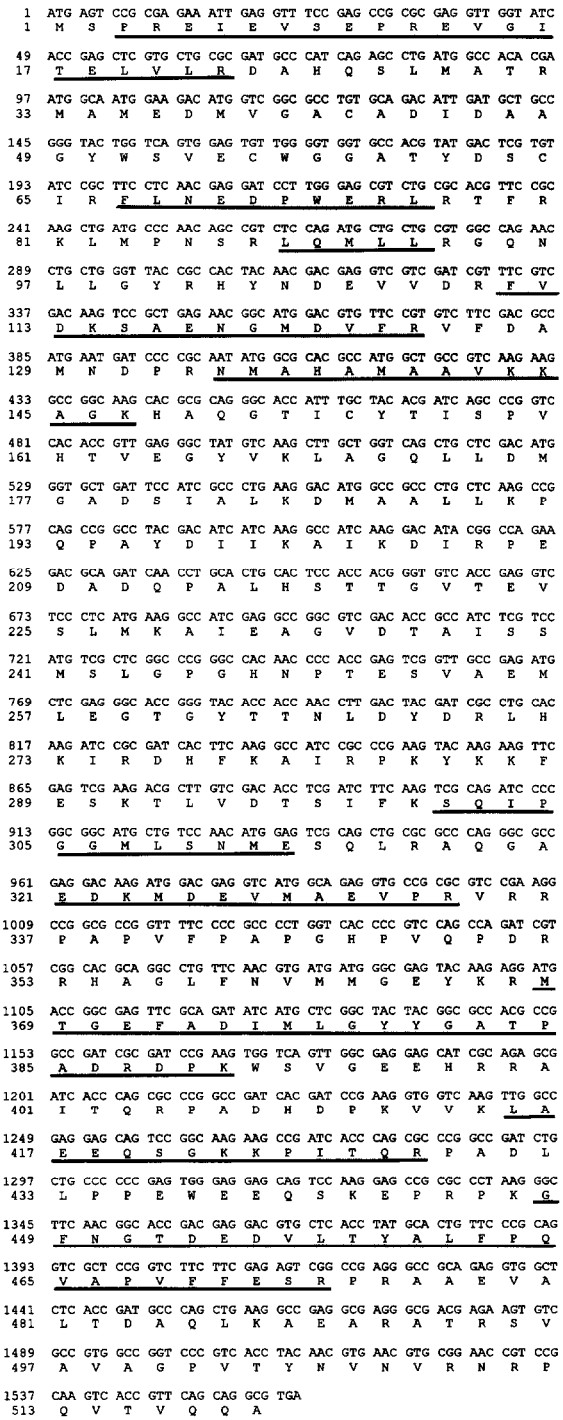

Fig. 1. Primary amino acid sequence and DNA sequence of the monomer of 5 S subunit of transcarboxylase. The underlined sequences are those peptides which were matched by amino acid sequencing. The GenBank accession number for this sequence is L06488.

3. RESULTS AND DISCUSSION

3.1. *Primary structure of the 5 S subunit*

A 7.1 kb *P. shermanii* genomic fragment containing the 1.3 S gene was isolated and partially sequenced. Two open reading frames upstream of the 1.3 S gene were identified. The 5′ most open reading frame encoded the gene for the monomer of the 5 S subunit. This conclusion was reached by matching the deduced amino acid sequence from the ORF with sequences generated from tryptic peptides of isolated, authentic 5 S. The DNA and protein sequence are shown in Fig. 1, the underlined sequences are those of peptides which were matched with the deduced sequence. One of the peptides was identified following *N*-bromosuccinimide modification of 5 S and matched residues 67–76. This peptide contains Trp-73 which is protected from modification by pyruvate and is presumably at or near the pyruvate binding site. The 5 S subunit DNA sequence contains 1560 nucleotides encoding a protein of 519 amino acids with a calculated molecular weight of 57,793 Da. Amino acid compositions of the authentic 5 S subunits and of the amino acid sequence deduced from DNA are in close agreement providing further confidence that the DNA sequence represents 5 S (data not shown).

3.2. *Expression of the 5 S subunit*

A 2.7 kb *Eco*RI fragment was subcloned into pTZ18 to express 5 S in *E. coli* strain HB101 (see section 2). Fig. 2 shows a Western blot of total cell extracts using anti-TC antiserum (panel A) and anti-5 S antiserum (panel B) as primary antibodies. An immunoreactive band which co-migrated with authentic 5 S was visible in cell extracts prepared from *E. coli* in which the presumed 5 S was positioned downstream from the lac promoter of the vector. The immunoreactive band reacts specifically with anti-5 S antiserum.

3.3. *Partial reaction*

To determine whether the expressed 5 S WT was capable of catalyzing Partial Reaction 2 in crude *E. coli* extracts, activity was measured using [4-^{14}C]oxalacetate as substrate as described in section 2. The results were compared with the authentic 5 S subunit from *P. shermanii* and are shown in Fig. 3. Both 5 S WT and *P. shermanii* 5 S were equally active in Partial Reaction 2. The results suggest that the 5 S WT can be expressed in an active form which is indistinguishable from *P. shermanii* 5 S subunit.

3.4. *Sequence comparison*

We [21] and others [22–25] have previously noted regions of striking sequence identity between 5 S and the α-subunit of oxalacetate decarboxylase and pyruvate carboxylase, enzymes which catalyze the same or the reverse of partial reaction 2. Fig. 4 shows a sequence comparison between the portions of these proteins from all species which have now been reported including a mammalian pyruvate carboxylase. Most of the sequence identity with 5 S is found over the N-terminal 294 amino acids shown in the figure. Over this large span, all five of the proteins in Fig. 4 are at least 34% identical. The identity between 5 S and *Salmonella ty-*

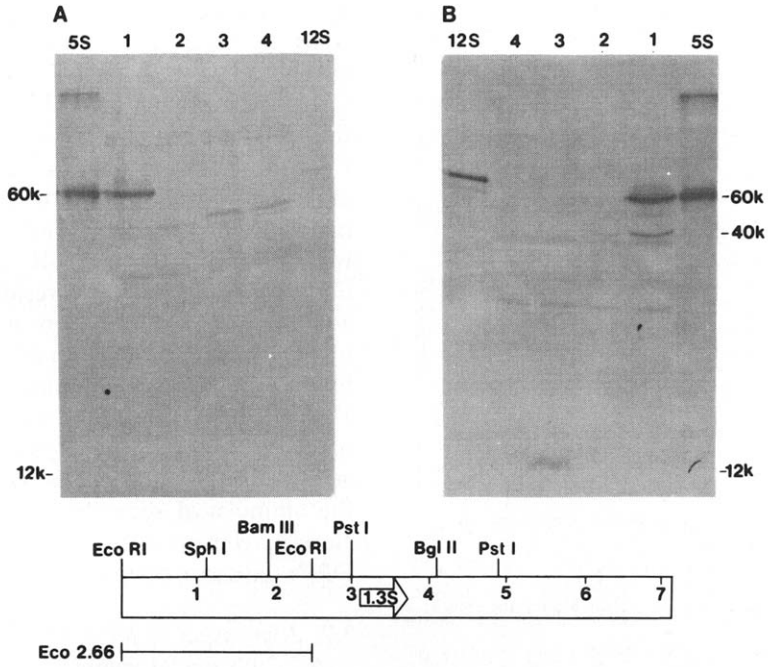

Fig. 2. Western blot analysis of the 5 S subunit expressed in *E. coli*. (A) With anti-5 S antiserum. 5 S, authentic 5 S from *P.shermanii*; lane 1, crude extract of *E. coli* HB 101 (plac5S); lane 2, crude extract of *E. coli* CSH26 as negative control; lane 3, crude extract of 1.3 S subunit expressed in CSH26 (ptac1.3); lane 4, crude extract of *E. coli* HB101 negative control; authentic 12 S from *P. shermanii*. (B) Western blot analysis as above with anti-TC antiserum.

phimurium oxalacetate decarboxylase over this region is 56%. There are two particularly strong regions of similarity extending from amino acid 49 to 77 in the 5 S sequence and amino acid 89 to 135. In these regions the percentage of identical amino acids increases to 55% and 47% respectively (79% and 83% for oxalacetate decarboxylase). We believe that one of these highly conserved regions to be at or near the pyruvate binding site. As noted earlier, we have identified Trp-73 as being associated with pyruvate binding. From Fig. 4 it is obvious that Trp-73 is in one of the most highly conserved regions of these proteins providing additional support to the hypothesis that this is a substrate binding site region. This region may also be important for metal coordination which is necessary for structural integrity [27]. The 5 S subunit must also have regions necessary for dimerization and interaction with the 1.3 S subunit. As interaction with a separate biotinyl subunit does not occur in oxalacetate decarboxylase or pyruvate carboxylase, we hypothesize that the COOH-terminus of the 5 S sequence is responsible for this function. The remainder of the sequence conservation over the entire N-terminal two-thirds of the 5 S protein appears to be necessary for catalysis, structure, metal coordination and multimerization.

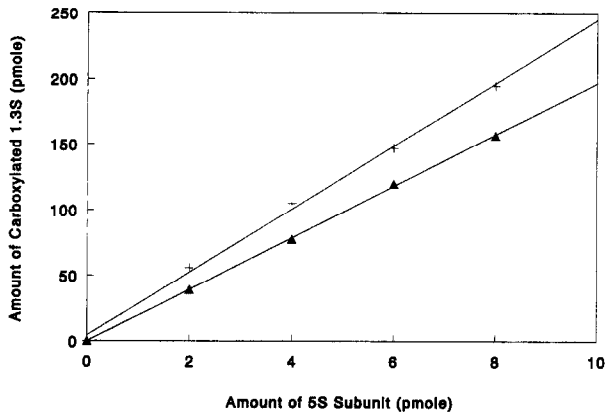

Fig. 3. Comparison of the activities of crude extract of *E. coli* 5 S WT with authentic *P. shermanii* 5 S subunit in partial reaction 2 of TC. Partial reaction 2 was carried out as described in section 2 with crude *E. coli* extract containing 5 S WT using [^{14}C]oxalacetate as substrate. The amount of 5 S subunit in the crude extract was quantitated by Western blot analysis through comparison with a 5 S standard titration. ▲, *E. coli* 5 S (crude extract); +, *P. shermanii* 5 S.

The results presented show conclusively that the 5 S subunit of *P. shermanii* transcarboxylase has been cloned and expressed in *E. coli*. The expressed 5 S subunit is fully active in the carboxylation of the 1.3 S biotinyl subunit with [4-^{14}C]oxalacetate. The availability of the cloned 5 S subunit now provides an opportunity to investigate the requirement for particular residues at the putative keto acid binding site, the binding site for the 1.3 S subunit, the residues involved in metal coordination and the site(s) involved in the tight linkage of the monomers to form the 5 S dimer.

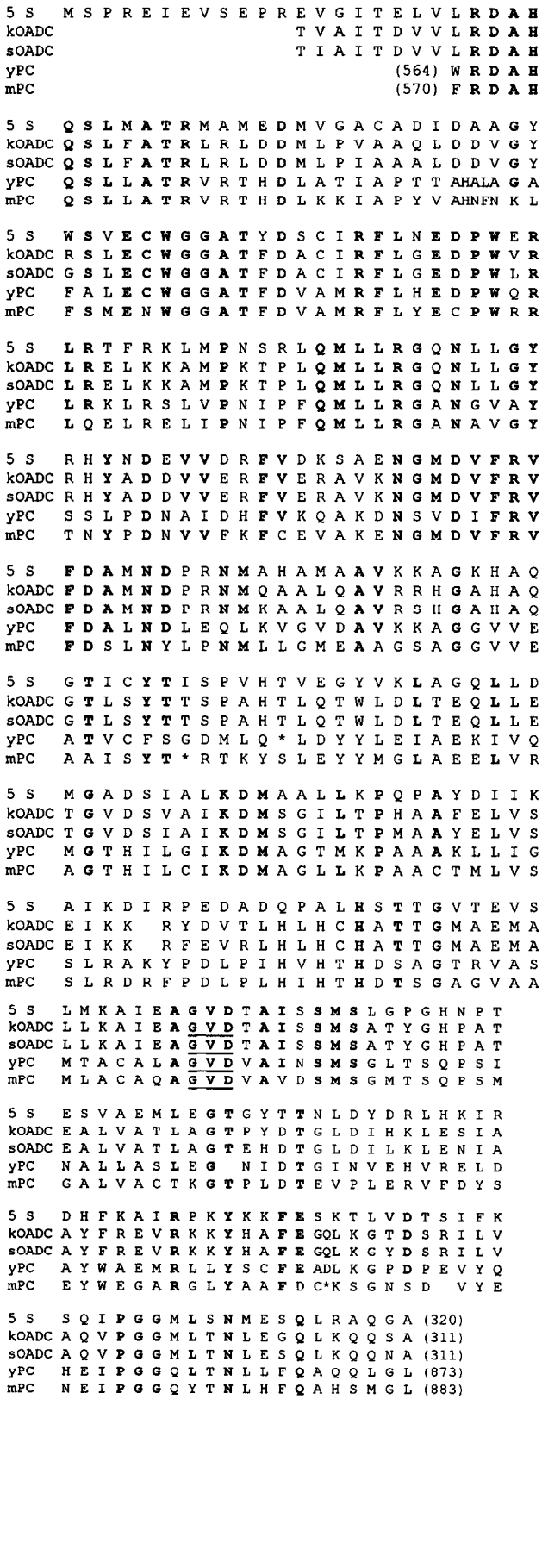

Fig. 4. Comparison of 5 S amino acid sequence with homologous sequences. The amino acid sequence of 5 S was compared to the GENPEPT database by BLASTP [26] through the NCBI BLAST Network service to identify proteins with sequence similarity. Once identified, those with more than 20% identity were aligned by the GCG Bestfit program. Underlines and asterisks indicate segments deleted to improve the alignment.__ represents 3 residues 'looped out', * represents 5. The sequences presented here are: 5 S, *P. shermanii transcarboxylase 5 S subunit; kOADC, Klebsiella pneumonia oxalacetate decarboxylase; sOADC, Salmonella typimurium; yPC, Saccharomyces cerevisiae pyruvate carboxylase; mPC, Mus musculus pyruvate carboxylase.*

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