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Phosphoglycerate Mutase from *Streptomyces coelicolor* A3(2): Purification and Characterization of the Enzyme and Cloning and Sequence Analysis of the Gene

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The enzyme 3-phosphoglycerate mutase was purified 192-fold from *Streptomyces coelicolor*, and its N-terminal sequence was determined. The enzyme is tetrameric with a subunit M_r of 29,000. It is 2,3-bisphosphoglycerate dependent and inhibited by vanadate. The gene encoding the enzyme was cloned by using a synthetic oligonucleotide probe designed from the N-terminal peptide sequence, and the complete coding sequence was determined. The deduced amino acid sequence is 64% identical to that of the phosphoglycerate mutase of *Saccharomyces cerevisiae* and has substantial identity to those of other phosphoglycerate mutases.

The enzymes of central metabolism in streptomycete species have been studied very little, despite the considerable commercial importance of these organisms as sources of antibiotics. Many fermentations are glucose based, and it is usually assumed that glucose is metabolized principally by the Embden-Meyerhoff pathway. However, to date, for *Streptomyces* spp., no enzyme of the pathway has been characterized fully and none of the genes of the pathway have been cloned and analyzed. As part of a program to study the genes and enzymes of central metabolism in *Streptomyces coelicolor*, we purified to apparent homogeneity the aromatic biosynthetic enzyme shikimate dehydrogenase. Our purified preparation of shikimate dehydrogenase showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an M_r of 29,000, but when the amino-terminal sequence analysis of this preparation was attempted, a 14-residue sequence was obtained which was in low yield (3%) compared with the estimated number of picomoles of protein presented to the sequencer. The sequence corresponded closely to the N-terminal sequences of a number of eukaryotic 3-phosphoglycerate mutases (EC 2.7.5.3). Apparently the shikimate dehydrogenase in the preparation could not be sequenced, but it contained phosphoglycerate mutase as a minor contaminant. This chance observation prompted us to characterize this important glycolytic enzyme for which there is no primary structure information from any bacterial source. In this paper we report the purification of 3-phosphoglycerate mutase from *S. coelicolor* A3(2), biochemical characterization of the enzyme and comparison with some other 3-phosphoglycerate mutases, cloning and sequencing of the gene, analysis of the coding region, and comparison of the deduced amino acid sequence with those of the enzymes from other species.

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MATERIALS AND METHODS

Reagents. Reactive Blue 2-Sepharose CL-6B and Reactive Red 120-Agarose (type 3000-CL) were obtained from Sigma Chemical Co., Poole, Dorset, United Kingdom; rabbit muscle phosphoglycerate mutase and 3-phosphoglycerate (grade 1) were obtained from Boehringer Corp., Lewes, East Sussex, United Kingdom. Restriction endonucleases, bacteriophage-T4 DNA ligase, T4 polynucleotide kinase, and the Klenow fragment of *Escherichia coli* DNA polymerase were purchased from Gibco-BRL, Paisley, Scotland, United Kingdom; Taq DNA polymerase and Taquence sequencing kits were from U.S. Biochemical Corp. via Cambridge Bioscience, Cambridge, United Kingdom.

Bacterial strains, vectors, and growth of cells for enzyme isolation. *S. coelicolor* JI3456 ($SCP1^{NF}$, $SCP2^-$) was provided by D. A. Hopwood, John Innes Institute. *E. coli* DS941 (23) and plasmid pUC18 were used in the primary genomic cloning. *E. coli* TG1 and phages M13 mp18 and M13 mp19 (18) were used for DNA sequencing. *S. coelicolor* JI3456 was grown in YEME medium (9).

Assay of 3-phosphoglycerate mutase activity. The enolase coupled assay was used with an assay volume of 1 ml (7). One unit is defined as a change in A_{240} of 0.1 per min. Under these conditions 1 μmol of 3-phosphoglycerate consumed per min is reported to be equivalent to a change in A_{240} of 0.87 (7). Protein was determined by the method of Sedmak and Grossberg (20) with bovine serum albumin as a standard.

Purification of 3-phosphoglycerate mutase. All steps in the purification of 3-phosphoglycerate mutase were performed at 4°C unless otherwise stated.

(i) Step 1: preparation of crude extract. A 20-g (wet weight) batch of *S. coelicolor* grown to the midlogarithmic phase for 48 h was harvested and resuspended in 25 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, 1.2 mM phenylmethylsulfonyl fluoride, and 0.4 mM dithiothreitol. The cells were broken by passage through a French pressure cell (98-MPa internal pressure). The cell lysate was centrifuged at 100,000 × *g* for 1 h.

(ii) Step 2: fractionation with $(NH_4)_2SO_4$. The supernatant of the crude extract (20 ml) was subjected to fractionation

with ammonium sulfate. The fraction that precipitated between 50% and 70% saturation contained the enzyme activity. It was dissolved in 2 ml of 10 mM Tris-HCl (pH 8.0) (buffer A) and dialyzed overnight against buffer A.

(iii) Step 3: chromatography with Cibacron-Blue Sepharose. The solution from step 2 was applied to a column (2.5 cm by 5.0 cm^2) of Cibacron-Blue Sepharose, previously equilibrated with buffer A. The phosphoglycerate mutase activity was not retained when the column was eluted with buffer A. Fractions (3 ml) containing enzyme activity were pooled.

(iv) Step 4: chromatography with Procion-Red Agarose. The enzyme solution from step 3 was applied to a column (12 cm by 0.8 cm^2) of Procion-Red Agarose (Reactive Red 120-agarose) previously equilibrated with buffer A. After the column was washed with 2 column volumes of buffer A, phosphoglycerate mutase activity was eluted by including 4 mM 2,3-bisphosphoglycerate (BPG) in buffer A. Fractions (3 ml) containing enzyme activity were pooled for long-term storage at 4°C. Elution from the Procion-Red Agarose column could also be achieved by using 5 mM 3-phosphoglycerate instead of BPG in buffer A.

Circular dichroism spectra. Circular dichroism spectra were recorded at 20°C on a Jasco J-600 spectrophotometer. The enzyme (0.1 mg/ml) was in 10 mM Tris-HCl (pH 8.0); the cell path length was 0.1 cm.

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (12) with 12% (wt/vol) polyacrylamide gels. Protein was detected by staining with Coomassie blue. A calibration curve was constructed by using the following M_r markers: bovine serum albumin (66,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,200).

Native M_r of phosphoglycerate mutase. The M_r of the enzyme was determined by gel filtration with a column (40 cm by 6.2 cm^2) of Sephacryl S-300 eluted with 0.1 M sodium phosphate buffer (pH 7.4). The following proteins were used as native M_r standards: lactate dehydrogenase (144,000), aspartate aminotransferase (92,000), bovine serum albumin (66,000), ovalbumin (43,000), trypsinogen (24,000), and RNase (14,000).

Determination of amino-terminal amino acid sequence. A sample of the purified 3-phosphoglycerate mutase was sequenced by B. Dunbar (University of Aberdeen) on an Applied Biosystems model 470A gas-phase sequencer as described by Russell et al. (17). The analysis gave a 25-residue sequence.

The protein sample, which contained shikimate dehydrogenase purified to apparent homogeneity by gel electrophoresis criteria, was sequenced by J. Young (ICI Pharmaceuticals) as described by White et al. (25). Whereas 200 pmol was presented to the analyzer, the initial yield (3%) indicated that the major protein species in the sample (shikimate dehydrogenase) was not being sequenced. Fourteen amino acids of a minor species were detected with a step yield of 94%.

Oligonucleotides. Oligonucleotides for screening of genomic DNA and genomic libraries and for primers in DNA sequencing were synthesized by V. Math, Department of Biochemistry, University of Glasgow, with an Applied Biosystems model 380A DNA synthesizer or an Applied Biosystems PCR-Mate.

Molecular biological methods. Total DNA of *S. coelicolor* JI3456 was prepared essentially as described by Hopwood et al. (9). Other molecular biological procedures were carried out as described by Sambrook et al. (18). Genomic digests were transferred to Hybond-N (Amersham) as described by Southern (22). The filter was incubated with radiolabeled oligonucleotide as described by Binnie (2). DNA sequencing was performed by the dideoxy-chain termination method (19) with [α-^{32}P]dATP. To overcome problems of primer extension associated with secondary structure of template DNA, sequencing reactions were carried out at 70°C with Taq DNA polymerase, usually with 7-deaza-dGTP as a replacement for dGTP in the reactions. Electrophoresis was in 8 M urea–6% (wt/vol) polyacrylamide linear gels. Sequences were compiled and analyzed by using the sequence analysis programs of the University of Wisconsin Genetics Computer Group (5).

Nucleotide sequence accession number. The data shown in Fig. 5 have been deposited with the EMBL data base under accession number X123456.

TABLE 1. Purification scheme for 3-phosphoglycerate mutase of *S. coelicolor*[a]

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
Crude extract	91	1,450	15	100
Ammonium sulfate	26	1,040	40	72
Cibacron-Blue chromatography	14.2	1,080	76	75
Procion-Red chromatography eluted with BPG	0.17	490	2,880	34

[a] These data are from cell samples of 20 g (wet weight).

RESULTS

Purification of enzyme. The purification of the 3-phosphoglycerate mutase of *S. coelicolor* is summarized in Table 1. The enzyme preparation was at least 95% homogeneous, as judged by SDS-PAGE (Fig. 1). The specific activity (2,880 U/mg) corresponded to a 192-fold purification.

Quaternary structure of enzyme. The subunit of 3-phosphoglycerate mutase migrated on SDS-PAGE gels with a

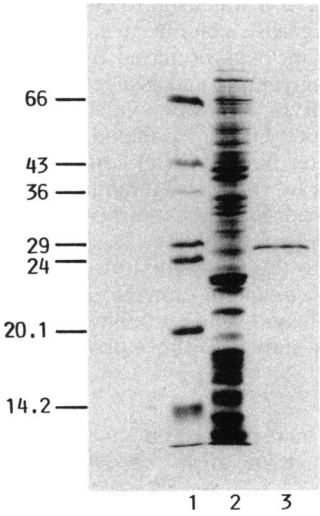

FIG. 1. SDS-PAGE of phosphoglycerate mutase at various stages of purification. Lanes: 1, molecular weight markers; 2, after elution through the Cibacron-Blue column; 3, after elution through the Procion-Red column. The numbered bars show the M_r values (10^3) of marker proteins.

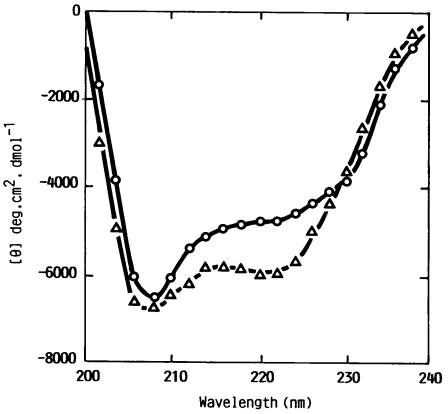

FIG. 2. Circular dichroism spectrum △, *S. coelicolor* enzyme; ○, *S. cerevisiae* enzyme (data from Hermann et al. [8]). For further details, see the text.

mobility consistent with an M_r of 28,800 ± 2,000. The native M_r, determined by gel filtration, was estimated at 120,000 ± 10,000. Taken together, these results indicate that the *S. coelicolor* enzyme is a tetramer, similar to that from *Saccharomyces cerevisiae*.

Circular dichroism spectrum. In a preliminary experiment, it was shown that the far-UV circular dichroism spectrum of the enzyme (Fig. 2) was similar in shape and magnitude to that determined previously for the *S. cerevisiae* enzyme (8). It would appear that the two enzymes have similar overall secondary structures, although this conclusion would have to be confirmed when larger quantities of the *S. coelicolor* enzyme are available.

Kinetic properties. (i) Dependence of enzyme activity on BPG. The enzyme from *S. cerevisiae* is dependent on BPG as a cofactor for full activity (10). After prolonged dialysis (5 days, 12 changes) against 10 mM Tris-HCl (pH 8.0), the enzyme from *S. coelicolor* was active in the absence of added BPG. This could have been due to the stability of the putative phosphoenzyme intermediate formed in the presence of BPG, which was used to elute the enzyme from the Procion-Red Agarose column at step 4 of the purification. When the enzyme was prepared by elution of the column with 3-phosphoglycerate and assayed in the absence of BPG, the specific activity was low (590 U/mg). The activity was restored (to 3,000 U/mg) by including 0.3 mM BPG in the assay (i.e., under normal assay conditions).

(ii) Inhibition by vanadate. Inhibition of phosphoglycerate mutases by vanadate has been proposed as a diagnostic tool for BPG-dependent enzymes (3). The addition of sodium metavanadate to the assay mixture resulted in marked inhibition of the *S. coelicolor* enzyme; 10 and 100 μM metavanadate led to 80 and 100% inhibition, respectively. These values are very similar to those observed by Carreras et al. (3) for a variety of BPG-dependent enzymes. The enzyme from *S. coelicolor* behaved similarly to others in that inhibition became fully effective only 2 to 3 min after the addition of metavanadate.

(iii) K_m for 3-phosphoglycerate. In the presence of 0.3 mM BPG, the K_m for 3-phosphoglycerate was 1.3 ± 0.1 mM, a value similar to those reported for other BPG-dependent enzymes under these assay conditions (16).

Cloning of the phosphoglycerate mutase gene. The minor protein species in the (apparently pure) shikimate dehydrogenase preparation gave the amino-terminal sequence ADAPYKLILRHG. By using the TFASTA program within the GCG DNA manipulation package, this peptide sequence was compared with all sequences in the GenBank data base (release 60). The streptomycete protein had high sequence identity with phosphoglycerate mutases. A fresh batch of cells was prepared, and phosphoglycerate mutase was purified as described above (Table 1). The N-terminal sequence of the preparation of pure enzyme confirmed the original data and extended it to 25 amino acids. Streptomycete genes have an unusual codon bias due to the high G+C composition (73%) of total DNA (1), which simplifies the design of oligonucleotide probes to clone genes based on peptide sequence data. A 24-nucleotide probe (24-mer) was designed against amino acids 1 through 9 of the protein (Fig. 3). The probe had two redundancies.

Digests of total DNA of *S. coelicolor* JI3456 were transferred (22) to nylon membranes. Conditions for hybridization and washing were varied until a unique signal of labeled probe was obtained. The optimum conditions were as follows: radiolabeled oligonucleotide (10 ng/ml; $>10^8$ dpm/μg) incubated for 1.5 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (18)–0.5% (wt/vol) sodium pyrophosphate–0.5% (wt/vol) SDS–200 μg of heparin per ml and then washed twice at 65°C in 5× SSC–0.5% (wt/vol) SDS. Of the various signals obtained with restriction digestions of genomic DNA with different enzymes, a 3.1-kb *Sal*I band was judged to be optimal for subcloning. A genomic sublibrary containing *Sal*I fragments from 2.8 to 3.5 kb in size was subcloned into the vector pUC18. Recombinants containing the hybridizing sequence were identified by colony hybridization; pGLW105 was taken as a representative recombinant.

DNA sequence. The genomic insert of pGLW105 was subcloned into M13 mp18 (to give mGLW106) and into M13 mp19 (to give mGLW107). DNA sequence was obtained (Fig. 4 gives the overall strategy) with a universal primer and with oligonucleotide primers constructed sequentially. The complete nucleotide sequence and deduced amino acid sequence are shown in Fig. 5.

DISCUSSION

The biochemical properties of phosphoglycerate mutase from *S. coelicolor* (subunit molecular weight, quaternary

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amino acid        1   2   3   4       5   6   7       8   9
 residue
                  A   D   A   P       Y   K   L       I   L

oligo     5'   C GAC GCC CC(CG) TAC AAG CT(GC) ATC CT     3'
               * *** **  **  *  *** *** **  *  *** **
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FIG. 3. Amino-terminal sequence of phosphoglycerate mutase and design of the oligonucleotide probe. Asterisks indicate bases of the probe that are identical to those of the genomic DNA sequence.

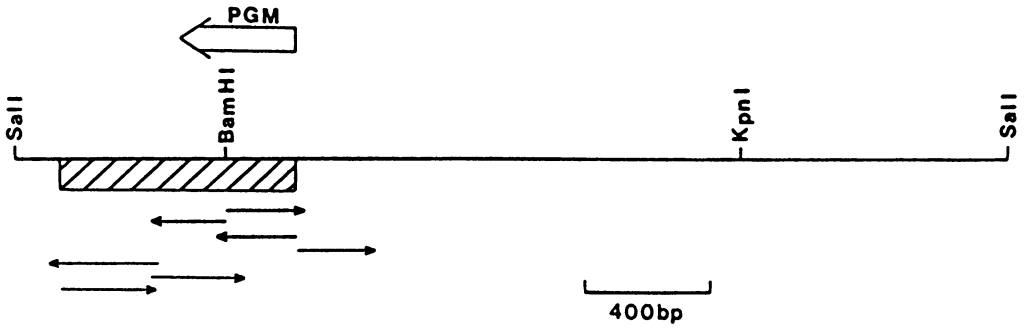

FIG. 4. Sequencing strategy for the phosphoglycerate mutase (PGM) gene.

structure, circular dichroism spectrum, cofactor or inhibitor dependence) implied that the enzyme was similar to that described previously from *S. cerevisiae* (10). At the level of deduced primary amino acid sequence, the *S. coelicolor* enzyme shows high similarity to the other phosphoglycerate mutases for which primary structures are available (Fig. 6). As far as we are aware, this is the first prokaryotic sequence to be reported. The sequence identity with phosphoglycerate

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   1  CCGTCCAACCGTCCGCCCACCGGGGCGCACGCGCGGGGGATCAGGCCTTGGATTACGCTC   60
  61  GGAAGCATGGCCGACGCACCGTACAAGCTGATCCTCCTCCGCCACGGCGAGAGCGAGTGG  120
      RBS  M  A  D  A  P  Y  K  L  I  L  L  R  H  G  E  S  E  W
 121  AACGAGAAGAACCTGTTCACCGGCTGGGTGGACGTCAACCTCACCCCGAAGGGCGAGAAG  180
      N  E  K  N  L  F  T  G  W  V  D  V  N  L  T  P  K  G  E  K
 181  GAGGCGACGCGCGGCGGCGAGCTGCTCAAGGACGCCGGCCTGCTGCCCGACGTGGTCCAC  240
      E  A  T  R  G  G  E  L  L  K  D  A  G  L  L  P  D  V  V  H
 241  ACGTCCGTCCAGAAGCGCGCGATCCGCACGGCCCAGCTCGCGCTGGAGGCCGCCGACCGC  300
      T  S  V  Q  K  R  A  I  R  T  A  Q  L  A  L  E  A  A  D  R
 301  CACTGGATCCCGGTCCACCGCCACTGGCGCCTGAACGAGCGCCACTACGGCGCCCTCCAG  360
      H  W  I  P  V  H  R  H  W  R  L  N  E  R  H  Y  G  A  L  Q
 361  GGCAAGGACAAGGCCCAGACCCTCGCCGAGTTCGGCGAGGAGCAGTTCATGCTGTGGCGC  420
      G  K  D  K  A  Q  T  L  A  E  F  G  E  E  Q  F  M  L  W  R
 421  CGCTCCTACGACACCCCGCCGCCCGCGCTGGACCGCGACGCCGAGTACTCCCAGTTCTCC  480
      R  S  Y  D  T  P  P  P  A  L  D  R  D  A  E  Y  S  Q  F  S
 481  GACCCGCGTTACGCGATGCTCCCGCCGGAGCTGCGCCCGCAGACGGAGTGCCTGAAGGAC  540
      D  P  R  Y  A  M  L  P  P  E  L  R  P  Q  T  E  C  L  K  D
 541  GTCGTCGGCCGGATGCTCCCGTACTGGTTCGACGCGATCGTCCCCGACCTCCTCACCGGC  600
      V  V  G  R  M  L  P  Y  W  F  D  A  I  V  P  D  L  L  T  G
 601  CGCACGGTCCTGGTGGCGGCGCACGGCAACTCCCTCCGCGCCCTCGTCAAGCACCTCGAC  660
      R  T  V  L  V  A  A  H  G  N  S  L  R  A  L  V  K  H  L  D
 661  GGCATCTCCGACGCCGACATCGCGGGCCTGAACATCCCGACGGGCATCCCGCTCTCGTAC  720
      G  I  S  D  A  D  I  A  G  L  N  I  P  T  G  I  P  L  S  Y
 721  GAACTCAACGCCGAGTTCAAGCCCCTGAACCCGGGCGGCACGTACCTCGACCCGGACGCG  780
      E  L  N  A  E  F  K  P  L  N  P  G  G  T  Y  L  D  P  D  A
 781  GCCGCGGCGGCGATCGAGGCCGTGAAGAACCAGGGCAAGAAGAAGTAAGCGCGCACGAAC  840
      A  A  A  A  I  E  A  V  K  N  Q  G  K  K  K  *
 841  AGGCCCCCTACCTGCGGTTTCTCCGCGAGTAGGGGGCTTTGTGTTGTCGTGGGCCGTCTC  900
 901  TGGGCCGTTTCTTGCTCGGCG  921
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FIG. 5. DNA sequence and deduced protein sequence.

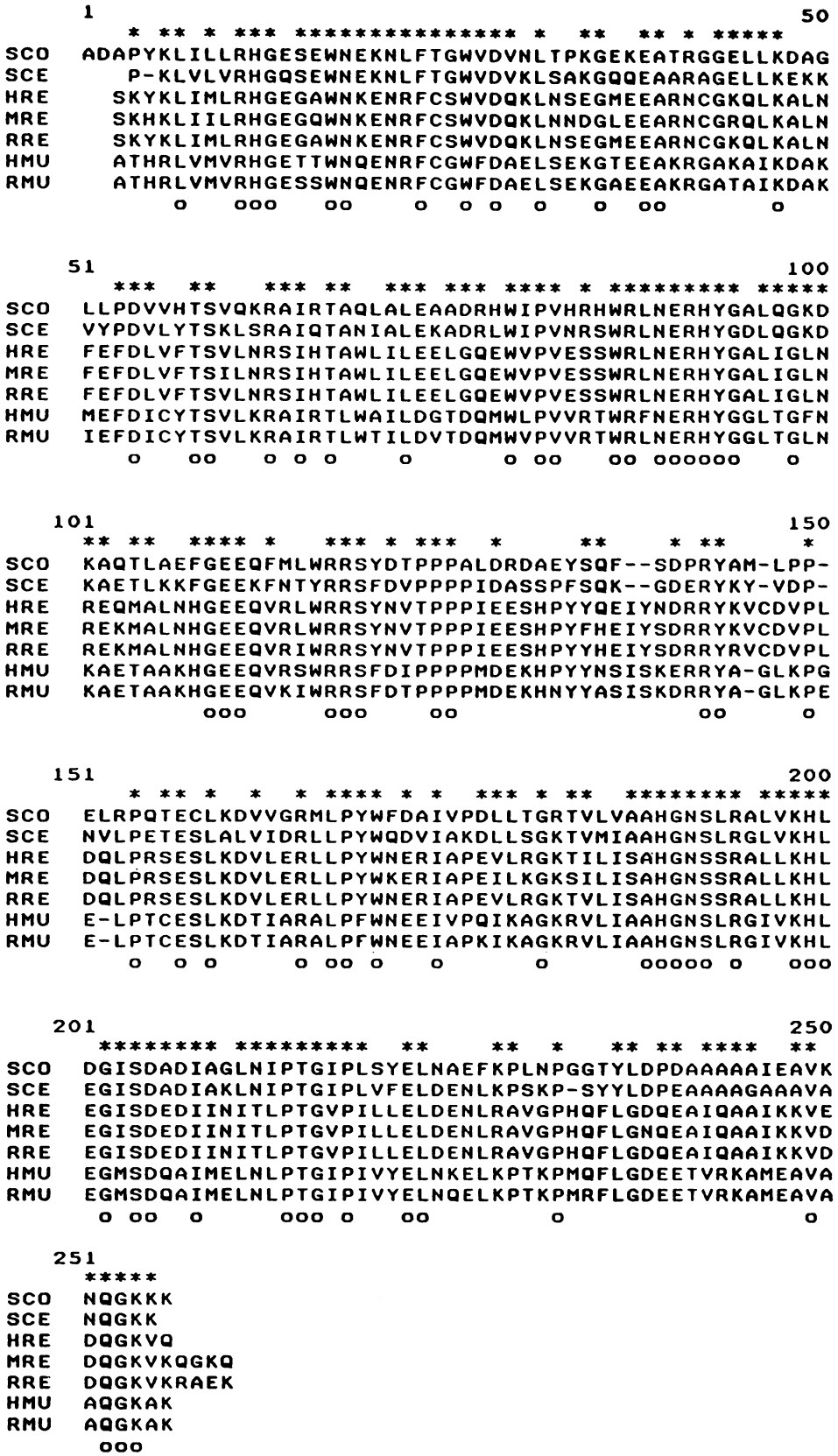

FIG. 6. Comparison of protein sequences of phosphoglycerate mutase from *S. coelicolor* (SCO) (this work), *S. cerevisiae* (SCE) (24), human reticulocyte (HRE) (11), mouse reticulocyte (MRE) (14), rabbit reticulocyte (RRE) (27), human muscle (HMU) (21), and rat muscle (RMU) (4). Sequences of the enzymes of *S. coelicolor* and *S. cerevisiae* that are identical (*) and by residues that are identical in all seven proteins (O) are indicated.

mutase from *S. cerevisiae* is particularly striking, but the high sequence identity with the mammalian proteins also reinforces the view (6) that glycolytic genes have been highly conserved during evolution. Of particular note, the *S. coelicolor* enzyme has at its carboxy terminus an unusual run of alanine residues and several lysines. The codons for lysine (AAA, AAG) are A+T rich. Because of the high G+C content of streptomycete DNA, lysine codons are relatively rare. Where there is a requirement for a positively charged residue, arginine (which has six codons, some of which contain only G+C) is often substituted for lysine (24a). The flexible tail of lysine residues, which is highly conserved between *S. coelicolor* and *S. cerevisiae*, has been proposed to be involved in limiting the access of substrate to the active site. The crystal structure of the *S. cerevisiae* enzyme has been determined (26), so it will be possible to use it to model the likely structure of the streptomycete protein.

Despite the high sequence identity with the other mutases, the enzyme from *S. coelicolor* has some unusual properties. It did not bind to Cibacron-Blue, a property previously correlated with dependence on BPG (16). Even after prolonged dialysis, it was difficult to demonstrate BPG dependence when the enzyme was eluted with BPG during the last step in purification (Table 1). It was only when the enzyme was eluted with 3-phosphoglycerate that BPG dependence could be demonstrated effectively. The difficulty in establishing this dependence implies either that the *S. coelicolor* binds BPG very tightly compared with the enzyme from other sources or that the putative phosphorylated form of the *S. coelicolor* enzyme is very stable toward hydrolysis. Inhibition was observed when vanadate was added, which is consistent with the proposed BPG dependence of the enzyme (3).

The DNA sequence revealed that the 24-mer oligonucleotide probe designed to clone the gene (Fig. 3) had only one mismatch. The predicted amino acid sequence was identical to the first 25 residues determined by sequencing the purified protein. In common with many bacterial proteins, the nascent polypeptide is processed to remove the *f*-methionyl residue to give the native form of the protein. A reasonable ribosome binding site (CGGA) was situated just upstream of the ATG start codon. The coding region of the gene displayed the G+C bias that is usual for streptomycete genes (1): 69% G+C in the first position, 42% G+C in the second position, and 99% G+C in the third position. By using the program Codonpreference (5) with threshold of 0.10, only two rare codons were identified within the coding region. Thus, the *S. coelicolor* gene shows the paucity of rare codons observed with glycolytic genes of other species, and this is likely to be a feature of other glycolytic genes from streptomycetes. Most of the streptomycete genes sequenced to date have been involved in differentiation or antibiotic biosynthesis and resistance. Expression of at least 1 tRNA species ($tRNA^{Leu}_{TTA}$ [13]) is regulated during the streptomycete life cycle, which results in temporal regulation of translation of genes containing TTA codons. The phosphoglycerate mutase gene of *S. coelicolor* has no TTA codons. It has also been suggested that antibiotic-related genes could have a codon usage that is different from those of central metabolism. The codon usage of the (highly expressed) phosphoglycerate mutase gene is not significantly different from those of other streptomycete genes sequenced so far, so this is unlikely to be the case. Although the peptide sequence translated from the DNA sequence of this gene corresponds at the amino terminus to the purified protein and has 64% identity with the corresponding protein of *S. cerevisiae*, it remains to be proven by overexpression or gene disruption that the cloned gene actually encodes the enzyme.

Under the growth conditions employed in this study, phosphoglycerate mutase was some 0.5% of the total protein of *S. coelicolor*. In other species many glycolytic genes are highly expressed and have formed the basis of high-level expression vectors. It will be important to identify and characterize the promoter of this gene, which could be useful in the expression of heterologous genes, and to study its activity during growth on glycolytic and gluconeogenic substrates.

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