Genetic and biochemical characterization of Salmonella enterica serovar typhi deoxyribokinase

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LISE TOURNEUX,1 NADIA BUCURENCI,1‡ COSMIN SAVEANU,1‡ PIERRE ALEXANDRE KAMINSKI,2 MADELEINE BOUZON,2 ELISABETH PISTOTNIK,1 ABDELKADER NAMANE,1 PHILIPPE MARLIERE,2 OCTAVIAN BĂRZU,1 INES LI DE LA SIERRA,1 JAN NEUHARD,4 AND ANNE-MARIE GILLES1*1

1Laboratoire de Chimie Structurale des Macromolécules,1 Unité de Biochimie Cellulaire,2 and Unité de Biochimie Structurale,3 Institut Pasteur, 75724 Paris Cedex 15, France, and Center for Enzyme Research,4 Institute of Molecular Biology, University of Copenhagen, Copenhagen K, Denmark

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We identified in the genome of Salmonella enterica serovar Typhi the gene encoding deoxyribokinase, deoK. Two other genes, vicinal to deoK, were determined to encode the putative deoxyribose transporter (deoP) and a repressor protein (deoQ). This locus, located between the uhpP1 and ilvN genes, is absent in Escherichia coli. The deoK gene inserted on a plasmid provides a selectable marker in E. coli for growth on deoxyribose-containing medium. Deoxyribokinase is a 306-amino-acid protein which exhibits about 35% identity with ribokinase from serovar Typhi, S. enterica serovar Typhimurium, or E. coli. The catalytic properties of the recombinant deoxyribokinase overproduced in E. coli correspond to those previously described for the enzyme isolated from serovar Typhimurium. From a sequence comparison between serovar Typhi deoxyribokinase and E. coli ribokinase, whose crystal structure was recently solved, we deduced that a key residue differentiating ribose and deoxyribose is Met10, which in ribokinase is replaced by Asn14. Replacement by site-directed mutagenesis of Met10 with Asn decreased the Vmax of deoxyribokinase by a factor of 2.5 and increased the Km for deoxyribose by a factor of 70, compared to the parent enzyme.

Deoxyribokinase (dRK) catalyzes the ATP-dependent phosphorylation of 2-deoxyribose to 2-deoxyribose-5-phosphate (dRib-5P). The enzyme was identified in bacterial species (Lactobacillus plantarum and Salmonella enterica serovar Typhimurium) capable of using deoxyribose as a sole carbon and energy source (6, 7, 8). The product of the reaction, dRib-5P, is subsequently cleaved to acetaldehyde and glyceraldehyde-3-phosphate (dRib-5P). The enzyme was identified in bacterial species (4, 16). The gene encoding dRK, deoK, is dRib-5P (8), indicating that expression of deoK is dRib-5P (4, 16). The gene encoding dRK, deoK, together with a locus encoding a putative deoxyribose per- mease (deoP), was tentatively mapped at about 20 min on the serovar Typhimurium chromosome (8). Expression of deoK is also inducible, but the inducer is free deoxyribose rather than dRib-5P (8), indicating that deoK is not part of the deo regulon. E. coli K-12 cannot grow with deoxyribose as the sole carbon source, presumably because it lacks dRK activity.

With the genome sequencing projects for S. enterica serovars Typhi, Typhimurium, and Paratyphi being under way, we aimed to identify the deoK gene locus in the released data bank by determining the N-terminal amino acid sequence of the partially purified enzyme from a nonpathogenic bacterial strain.

MATERIALS AND METHODS

Chemicals. Nucleotides, restriction enzymes, T4 DNA ligase, T7 DNA polymerase, and coupling enzymes were from Boehringer Mannheim. Vent DNA polymerase was from New England Biolabs. Oligonucleotides were synthesized by the phosphomimetic method, using a commercial DNA synthesizer (Cy- clone Biosource).

Bacterial strains, plasmids, growth conditions, and DNA manipulations. E. coli MG1655, NM554 (14), and BL21 (DE3) (22) strains were used for complementation, cloning experiments, and recombinant protein overproduction, respectively. The deoK gene from serovar Typhi strain Ty2 was amplified by PCR using the following upstream and downstream primers: 5′-GGCAAGCTTTTGTGACGATTTCGGAAGGGAG and 5′-CCCAAGCTTTATTCGTTCAACGAAAGATGACATCTATTAGAGGAGGAG and 5′-TAGGATCTTGACGCCGGACCT. The resulting 2.3-kb amplicon was cut with HindIII (at the sites underlined in the primer sequences) and ligated with the plasmid pSU19 (2) cut with the same enzyme. The deoKP region from serovar Typhi strain Ty2 was similarly amplified by PCR using the upstream and downstream primers 5′-GCAGCTTTTGACGGATTCCGGAAGGGAGGAG and 5′-TAGGATCTTGACGCCGGACCT. The resulting 2-kb amplicon was cut with HindIII and BamHI (at the sites underlined in the primer sequences) and ligated with the plasmid pSU19 cut with the same enzymes. The ribk gene from serovar Typhi- murium was amplified by PCR using the upstream and downstream primers 5′-CAGGATCTCAAGCCGATATGGAAACCCCGC and 5′-CCGAATTCTAAGCGTTTACCCCTGC. The resulting 0.9-kb amplicon was cut with BamHI and EcoRI (at the sites underlined in the primer sequences) and ligated with plasmid pSU19 cut with the same enzymes. The three recombinant plasmids were introduced in the E. coli strain MG1655 by calcium chloride transformation. Mineral medium MS5 (15) supplemented with 20 mM deoxyribose and solidified with 15 g of agar per liter was used to test the recombinant strains.

For recombinant protein overproduction, the deoK gene from serovar Typhi strain Ty2 was amplified by PCR. The two synthetic upstream and downstream oligonucleotides used for amplification were 5′-GGGGGAATTCAAGAAGGAATTTCTGAGAG and 5′-CCCAAGCTTTATTCGTTCAACGAAAGATGACATCTATTAGAGGAGGAG and 5′-TAGGATCTTGACGCCGGACCT. The resulting 2.3-kb amplicon was cut with HindIII and BamHI (at the sites underlined in the primer sequences) and ligated with the plasmid pSU19 cut with the same enzymes. The three recombinant plasmids were introduced in the E. coli strain MG1655 by calcium chloride transformation. Mineral medium MS5 (15) supplemented with 20 mM deoxyribose and solidified with 15 g of agar per liter was used to test the recombinant strains.

* Corresponding author. Mailing address: Laboratoire de Chimie Structurale des Macromolécules, Institut Pasteur, 28, rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: 33 (1) 45 68 89 68. Fax: 33 (1) 46 61 39 63. E-mail: amgilles@pasteur.fr.

† Permanent address: Laboratoire de Chimie Structurale des Macromolécules, Institut Pasteur, 75724 Paris Cedex 15, France, and Center for Enzyme Research, Institute of Molecular Biology, University of Copenhagen, Copenhagen K, Denmark.

‡ Permanent address: Department of Biochemistry, University of Medicine and Pharmacy, Cluj-Napoca, Romania.
GAGCCAATAAC-3’ (modified codon is in boldface). The sequence of the wild-type and of the mutated deoK gene was verified by the dideoxynucleotide sequencing method (18). For overexpression, plasmids pH15510 and pPFK511 (harbouring the mutated deoK gene) were introduced into the E. coli strain BL21 (DE3)/pRAB17.

Analytical methods. dRK activity was determined according to the method of Schimmel et al. (19) by coupling dRib-SP formation to NADH oxidation using dRib-SP aldolase and glycerophosphate dehydrogenase-triose phosphate isomerase as coupling enzymes or by coupling ADP formation to NADH oxidation using dRib-5P aldolase and glycerophosphate dehydrogenase—triose phosphate isomerase as coupling enzymes or by coupling ADP formation to NADH oxidation during regeneration of ATP by phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase. The reaction medium (0.5-ml final volume) contains 50 mM Tris-HCl (pH 7.4); 10 mM MgCl₂; 0.2 mM NADH; 1 mM ATP; 200 mM ammonium chloride; 1 U each of dRib-SP aldolase, glycerophosphate dehydrogenase, and triose phosphate isomerase or 50 mM Tris-HCl (pH 7.4); 10 mM MgCl₂; 0.2 mM NADH; 1 mM ATP; 1 mM phosphoenolpyruvate; 200 mM ammonium chloride; and 1 U each of pyruvate kinase and lactate dehydrogenase.

The reaction was started in each case with dRK at appropriate dilutions with 50 mM Tris-HCl (pH 7.4) and 2 mM d-ribose (or 10 mM d-ribose in the second assay), and the absorption decrease at 334 nm was monitored with an Eppendorf PCP6121 photometer with the thermostat set at 30°C. Protein concentration was measured according to the method of Bradford (3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (12). The protein bands from SDS-PAGE were electroblotted onto a Problot membrane filter (Applied Biosystems) and detected by staining with Coomassie blue. The N-terminal amino acid sequences of protein from excised bands were determined by a protein sequencer (Applied Biosystems, Inc.). Ion-trap mass spectra were recorded on an API 365 triple-quadrupole mass spectrometer (Perkin-Elmer-Scientific, Thornhill, Canada). Samples dissolved in water-methanol-formic acid (50/50/5 [vol/vol/vol]) were introduced at 5 μl/min into a spray pump (Harvard Apparatus, South Natick, Mass.). The mass spectra were stored continuously from 500 to 1,700 with a scan step of 0.1 and a dwell time per step of 2.0 ms, resulting in a scan duration of 16.0 s. Data were collected on a Power Macintosh 8600/200 and processed through the BioToolbox 2.2 software from Sciex.

RESULTS AND DISCUSSION

N-terminal sequence of dRK. Strain SL35 of serovar Typhimurium was grown in medium containing Casamino Acids (19). When optical density at 600 nm reached 1.5, the medium was supplemented with deoxyribose (1 g/liter), and the growth was continued for another hour. Bacteria collected for 30 min by centrifugation at 10,000 × g and 4°C were tested for dRK activity in the sonicated extract. The specific activity of dRK in crude extract (0.12 U/mg of protein) indicated that it represented ~0.15% of total bacterial proteins. The specific activity of dRK was 50-fold enriched by loading 100 mg of proteins onto a 6- by 1.5-cm Blue-Sepharose column equilibrated with 50 mM Tris-acetate (pH 6.0). dRK collected in the flow-through was immediately loaded onto a 4- by 1.5-cm DEAE- Sephacel column equilibrated with the same buffer. The column was washed with 0.2 M NaCl in 50 mM Tris-acetate (pH 6.0), and dRK was eluted with 0.5 M NaCl in the same buffer. The enzyme (6.4 U/mg of protein) identified by SDS-PAGE as the 60 N-terminal amino acid residues MDIAVIGSNMVDLITYTNQMPKEGETLEAPAFKIGCGGKGANQAVAA and the N-terminal amino acid sequence of dRK.

The 60 N-terminal amino acid residues MDIAVIGSNMVDLITYTNQMPKEGETLEAPAFKIGCGGKGANQAVAA were supplemented with deoxyribose (1 g/liter), and the growth of E. coli strain, which lacks dRK activity, was monitored continuously at 334 nm until the optical density at 600 nm reached 0.15% of total bacterial proteins. The specific activity of dRK in crude extract (0.12 U/mg of protein) indicated that it represented ~0.15% of total bacterial proteins. The specific activity of dRK was 50-fold enriched by loading 100 mg of proteins onto a 6- by 1.5-cm Blue-Sepharose column equilibrated with 50 mM Tris-acetate (pH 6.0), and dRK was eluted with 0.5 M NaCl in the same buffer. The mass spectra were recorded on an API 365 triple-quadrupole mass spectrometer (Perkin-Elmer-Sciex, Thornhill, Canada). Samples dissolved in water-methanol-formic acid (50/50/5 [vol/vol/vol]) were introduced at 5 μl/min into a spray pump (Harvard Apparatus, South Natick, Mass.). The mass spectra were stored continuously from 500 to 1,700 with a scan step of 0.1 and a dwell time per step of 2.0 ms, resulting in a scan duration of 16.0 s. Data were collected on a Power Macintosh 8600/200 and processed through the BioToolbox 2.2 software from Sciex.

FIG. 1. Alignment of amino acid sequences of dRK from serovar Typhi and RK from serovars Typhi and Typhimurium and E. coli using CLUSTAL W (23). Strictly conserved residues, expressed in one-letter code, are in boldface. Residues from E. coli RK identified in binding of ribose are marked by asterisks. The numbering above the alignment corresponds to the E. coli sequence.

Coding the fucose permease of E. coli. We hypothesized that this putative permease corresponds to the 2-keto-3-deoxyribose transporter described by Hofee (8) and named the corresponding gene deoP. The deoP gene is followed by an ORF encoding a protein of 337 amino acid residues with no equivalent in E. coli and by the uhpa and uhpb genes. In the 154-bp intergenic segment between the unknown gene and the uhpb gene, we identified a putative rho-independent terminator of the deoP genes, overlapping the putative uhpaAB promoter. This intergenic space as well as the identified promoter and terminator is different from those described for serovar Typhimurium (9). The 4,384-bp DNA fragment inserted between the uhpaA and uhvN genes containing deoK, deoP, and deoQ may represent a case of horizontal gene transfer. On the genetic maps of E. coli and serovar Typhimurium, uhpaA and uhvN are located at approximately 83 min. Assuming that deoK and deoP in serovar Typhi are located between uhpaA and uhvN, the previously reported position of deoK and deoP at 20 min on the serovar Typhimurium chromosome most likely is wrong.

deoK, a selectable genetic marker in E. coli. deoK from serovar Typhi could be used as a selective marker in E. coli. We identified a putative permease (Fig. 1) on December 6, 2016 by INSTITUT PASTEUR-Bibliothèque http://jb.asm.org/ Downloaded from
Interestingly, overexpression of the putative RK gene \textit{rbsK} from serovar Typhimurium strain LT2 also allowed growth of \textit{E. coli} with deoxyribose (Fig. 3B). Considering the high homology between serovar Typhi and serovar Typhi- 
turium \textit{rbsK} and serovar Typhi \textit{deoK} and \textit{rbsK} gene products (Fig. 1), this result favors a scenario of evolutionary improvement of a marginal dRK activity from an RK enzyme. 

Overproduction of serovar Typhi dRK. Plasmid pBLT5510 harboring the \textit{deoK} gene from serovar Typhi was introduced into the \textit{E. coli} strain BL21(DE3)/pDIA17. This strain (22) expressed the \textit{lacI} gene on plasmid pDIA17 (13) and the T7 RNA polymerase gene on the chromosome. Overproduction was carried out by growing bacteria at 37°C in 2YT medium (17) supplemented with kanamycin (50 \text{mg/ml}) and chloramphenicol (30 \text{mg/ml}). When the optical density at 600 nm reached 1.5, isopropyl-\textbeta-D-thiogalactoside (final concentration, 1 mM) was added to the medium. The bacteria were harvested by centrifugation 3 h after induction. SDS-PAGE followed by Coomassie blue staining indicated that recombinant dRK represented over 20\% of total bacterial proteins (Fig. 4). The molecular mass of dRK measured by electrospray ionization mass spectrometry (33,228.14 \pm 2.18 Da) was in agreement with that calculated (33,229.08) from the sequence. The recombinant dRK phosphorylated both deoxyribose and D-ribose with nearly identical \( V_{\text{max}} \) values. However, the \( K_{\text{m}} \) for deoxyribose (0.1 mM) was considerably lower than that for ribose (2 mM). These values were similar to those obtained with partially purified enzyme from serovar Typhimurium. 

Structure-function relationship of serovar Typhi dRK. The molecular mass of native dRK corresponds to a dimer (19), as is the case for the \textit{E. coli} RK and possibly for the serovar Typhi 

![Fig. 2. Organization of the \textit{deoK} region in the serovar Typhi genome and comparison with the equivalent region on \textit{E. coli} genome. Boxes correspond to genes, and numbers below boxes indicate the size of the gene product (number of amino acid residues). Horizontal arrows indicate the directions of transcription, and the vertical arrow locates the deleted locus on the genome of \textit{E. coli}.](image)

![Fig. 3. Acquisition of deoxyribose-catabolizing genes by \textit{E. coli}. The figure shows the growth pattern on deoxyribose (20 mM)-containing plates after 3 days at 37°C of \textit{E. coli} K-12 strain MG1655 transformed with the plasmid pSU19 bearing various inserts: none (A), the \textit{rbsK} gene amplified from serovar Typhimurium strain LT2 and encoding a putative RK (B), the \textit{deoK} gene amplified from serovar Typhi and encoding dRK (C), or the \textit{deoKP} locus amplified from serovar Typhi and encoding both dRK and a putative deoxyribose permease (D).](image)

![Fig. 4. SDS-PAGE (12.5% polyacrylamide) analysis (left panel, lane 2) and electrospray ionization mass spectrum (right panel) of a bacterial extract overproducing dRK from serovar Typhi. The molecular weight markers for SDS-PAGE analysis (lane 1) are, from top to bottom, phosphorylase \( a \) (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and lysozyme (14,400) (A to F, respectively).](image)
enzyme. The crystal structure of E. coli RK, recently solved at 1.8-Å resolution (20, 21), shows that the substrate ribose is maintained in the binding site by an intricate network of H bonds. Seven residues conserved among the RKs from E. coli, serovar Typhi, Haemophilus influenzae, and Schizosaccharomyces pombe are involved in this H-bond network binding (Fig. 1). Six of them (Asp16, Gly42, Lys43, Asn46, Glu143, and Asp255) exist in the sequence of serovar Typhi dRK while the Asn14 is replaced by a methionine residue (Met10). This methionine is also present in a probable dRK from serovar Typhimurium. The presence of a methionine instead of an asparagine residue lowers the strength of ribose binding to dRK compared to that to RK because of the loss of two H-bond interactions (involving O-1' and O-2' ribose atoms) (Fig. 5).

Moreover, the Met10 side chain and substrate positions are quite different when the three-dimensional structure models of dRK-ribose and dRK-deoxyribose are compared (Fig. 5, left). The methionine side chain should move to accommodate the hydroxyl linked to the ribose C-2 atom, and the ribose is moved aside from the Met10 residue in the dRK-ribose model. This difference may thus explain why dRK exhibits a better $K_m$ value for deoxyribose ($K_m = 0.1$ mM) than for ribose ($K_m = 2$ mM). To check this hypothesis, we replaced, by site-directed mutagenesis, Met10 in dRK with an Asn residue. The resulting protein has a molecular mass of 33,209.52 ± 1.86 Da, in agreement with that calculated (33,211.99) from the sequence. The Met10 Asn variant of dRK exhibits ~40% of the $V_{max}$ value of the wild-type protein with both deoxyribose and ribose as substrates. The $K_m$ for deoxyribose was 70-fold higher in the modified dRK compared to the parent enzyme, while under the same conditions the $K_m$ for ribose was only twofold increased in the Met10Asn variant compared to the wild-type dRK.

**Concluding remarks.** The various genome sequencing projects revealed that half of the putative proteins encoded by the newly found genes have no known function. On the other hand, well-characterized proteins isolated in the pregenomic era (almost 100 in the case of E. coli) are awaiting assignment to their corresponding genes. Partial purification of these proteins, coupled to gel electrophoresis and N-terminal sequencing or/and mass spectrometric analysis, might allow rapid and unequivocal assignment of a gene to a protein, as described in this work.

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