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NOTES

Cloning and Expression of the Two Genes Coding for L-Serine Dehydratase from *Peptostreptococcus asaccharolyticus*: Relationship of the Iron-Sulfur Protein to Both L-Serine Dehydratases from *Escherichia coli*

ANTJE E. M. HOFMEISTER,† SUSANNE TEXTOR, AND WOLFGANG BUCKEL*

Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität Marburg, Marburg, Germany

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The structural genes *sdhA* and *sdhB*, coding for the α- and β-subunits of the [4Fe-4S] cluster containing L-serine dehydratase from *Peptostreptococcus asaccharolyticus*, have been cloned and sequenced. Expression of modified *sdhB* together with *sdhA* in *Escherichia coli* led to overproduction of active His_6-tagged L-serine dehydratase. *E. coli* MEW22, deficient in the L-serine dehydratase L-SD1, was complemented by this *sdhBA* construct. The derived amino acid sequence of SdhBA shares similarities with both monomeric L-serine dehydratases, L-SD1 and L-SD2, from *E. coli* and with a putative L-serine dehydratase from *Haemophilus influenzae*, which suggests that these three enzymes are also iron-sulfur proteins.

L-Serine dehydratases and L-threonine dehydratases catalyze the irreversible overall deaminations of L-serine to pyruvate and L-threonine to 2-oxobutyrate. Most L-threonine dehydratases have been shown to contain pyridoxal-5′-phosphate as the prosthetic group. In contrast to L-threonine dehydratases, none of the bacterial L-serine dehydratases investigated to date has been conclusively proven to be dependent on pyridoxal-5′-phosphate (6). An L-serine dehydratase unequivocally devoid of pyridoxal-5′-phosphate was discovered in the gram-positive anaerobe *Peptostreptococcus asaccharolyticus* (clostridial cluster XIII) (2) and characterized as an iron-sulfur protein (5). This enzyme was inactivated by exposure to air and could be specifically reactivated by incubation with Fe^{2+} under anaerobic conditions. The dehydratase contains stoichiometric amounts of non-heme iron and acid-labile sulfur sufficient to form one $[4Fe\text{-}4S]^{2+}$ cluster per heterodimer (α, 30 kDa; β, 25 kDa). Electron paramagnetic resonance spectroscopic investigations supported the notion that the [4Fe-4S] cluster is involved in substrate binding and in catalysis by facilitating the elimination of the hydroxyl group of L-serine by a mechanism similar to that described for aconitase (8).

Cloning and sequencing of *sdhA* and *sdhB*. A preliminary report of part of this work has been published elsewhere (9). In an attempt to obtain a homologous probe for cloning, we derived four heterologous oligonucleotides from the N-terminal sequences of the α- and β-subunits of L-serine dehydratase, two in the forward (5′ → 3′) direction and two in the backward direction. They were used to prime a PCR (17) from chromosomal DNA of *P. asaccharolyticus* ATCC 14963, which was isolated as described by Marmur (12). Only the oligonucleotide combination β-forward and α-backward yielded a PCR product (0.75 kb), which indicated that the gene coding for the β-subunit (*sdhB*) preceded that coding for the α-subunit (*sdhA*) and that the amplified DNA was part of *sdhB*. This was confirmed by direct sequencing (1), since the N terminus of the derived amino acid sequence was identical to that of the β-subunit.

Southern blots (18) revealed strong hybridization of the digoxigenin-labeled *sdhB* probe with a single 3.6-kb *Pst*I fragment and a single 4.2-kb *Hin*dIII fragment of *P. asaccharolyticus* chromosomal DNA. Both fragments were chosen to establish size-selected libraries in the vector pBluescript SK^+ (Stratagene, Heidelberg, Germany), since they were likely to contain complete copies of *sdhA* and *sdhB*, which had been estimated at 1.65 kb on the basis of the molecular mass (55 kDa) of the combined heterodimeric L-serine dehydratase (5). For cloning procedures, *Escherichia coli* DH5α (7) was used. Five recombinant plasmids were confirmed to contain a 3.6-kb *Pst*I fragment which carried the whole *sdhB* gene and about 80% of *sdhA*. By the same method, one recombinant plasmid was demonstrated to contain a 4.2-kb *Hin*dIII fragment which carried complete copies of both *sdhA* and *sdhB*.

A region on the *Hin*dIII fragment of almost 2.3 kb in length was sequenced (19) in both directions. The DNA sequence is shown in Fig. 1, in which *sdhB* and *sdhA* represent open reading frames of 669 and 879 bp encoding the β- and α-subunits of L-serine dehydratase, respectively. The DNA sequence revealed not only that *sdhB* precedes *sdhA* in the same orientation but that both genes occur in the same reading frame, directly adjacent to each other. Putative ribosome binding sites for translation initiation were found 6 bp upstream of *sdhB* and 5 bp upstream of *sdhA* in the coding region of *sdhB* (Fig. 1). The N-terminal sequences of the α- and β-subunits of L-serine dehydratase and their respective calculated molecular masses of 30,774 and 24,154 Da are in good agreement with the data reported previously for the enzyme after purification from *P. asaccharolyticus* (5). As expected, no known consensus se-

* Corresponding author. Mailing address: Philipps-Universität Marburg, Fachbereich Biologie, Laboratorium für Mikrobiologie, D-35032 Marburg, Germany. Phone: 49 6421-281527. Fax: 49 6421-288979. E-mail: buckel@mailer.uni-marburg.de.

† Present address: Biological Laboratories, Harvard University, Cambridge, MA 02138-2097.

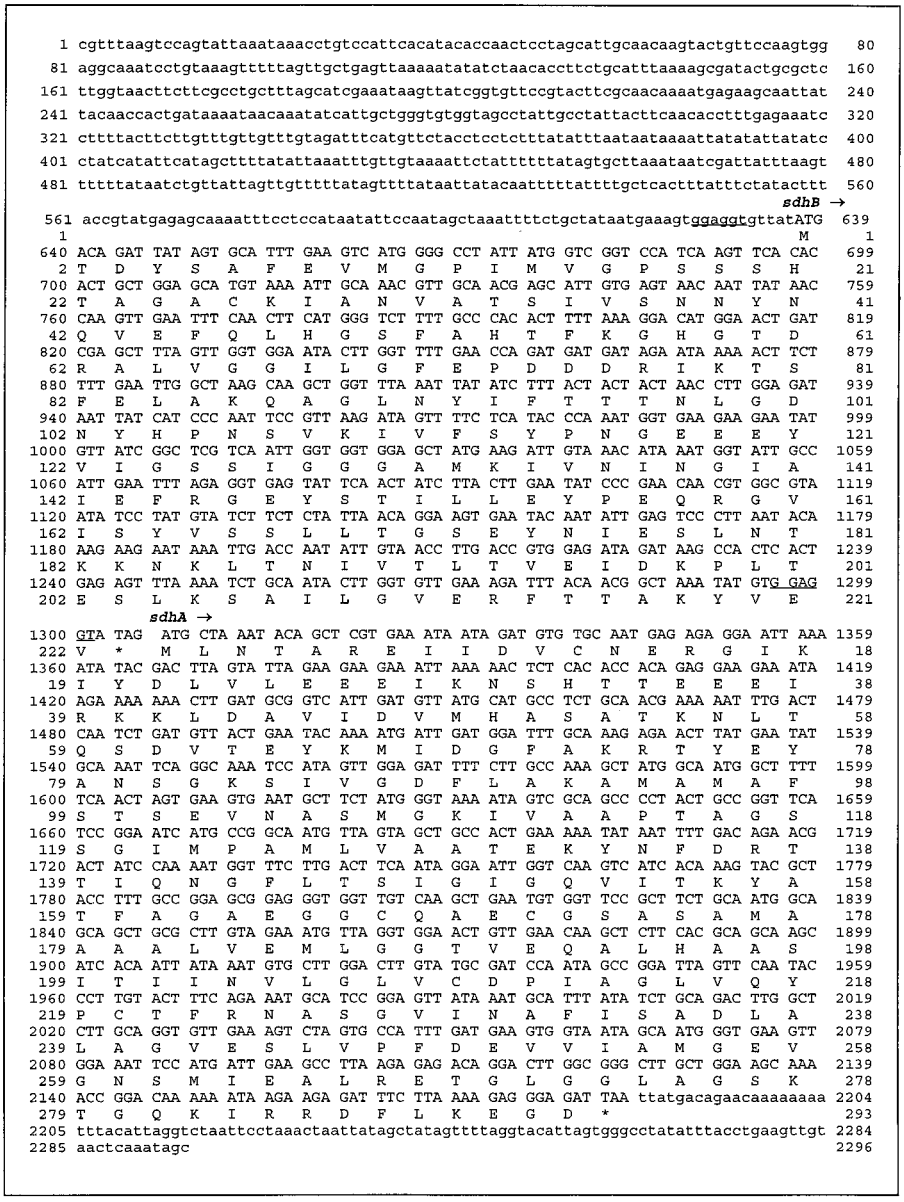

FIG. 1. Sequences of the two genes, *sdhA* and *sdhB*, coding for both subunits of L-serine dehydratase from *P. asaccharolyticus*. The determined DNA sequences and the deduced amino acid sequences of the two subunits are shown. The two genes occur in the same reading frame, directly adjacent to each other. Putative ribosome binding sites for translation initiation are underlined.

quence from pyridoxal-5′-phosphate-dependent enzymes (15, 16) was detectable.

Similarity of the amino acid sequences deduced from *sdhA* and *sdhB* with both L-serine dehydratases from *E. coli*. A comparison of the deduced amino acid sequences of *sdhA* and *sdhB* with all the sequences available in the nonredundant protein database by BLAST network service revealed identities with the two L-serine dehydratases present in *E. coli* (20, 22) and a putative L-serine dehydratase from *Haemophilus influenzae* (Fig. 2). This putative L-serine dehydratase was identified by 68% identity of the deduced amino acid sequence with those of the two L-serine dehydratases from *E. coli*, L-SD1 and L-SD2 (3). Each of these three proteins consists of only one subunit, which is nearly as large as the two subunits of L-serine dehydratase from *P. asaccharolyticus* together. They all showed 24% overall identity to SdhBA of *P. asaccharolyticus*. There is, however, an extended central region (amino acids N102 to V220 of the β-subunit and M1 to A99 of the α-subunit, i.e., M221 to A319 in Fig. 2) in the *P. asaccharolyticus* enzyme which has no counterpart in these three other enzymes. In the C-terminal part of SdhA, the α-subunit of the *P. asaccharolyticus* enzyme, three conserved cysteines were found (Fig. 2), which should be sufficient for the coordination of the [4Fe-4S] cluster as postulated for the mechanism of the enzyme (8).

Expression of *sdhA* and *sdhB* in *E. coli*. Cloned copies of *sdhA* and *sdhB* were placed under the control of inducible T7 promoter on vector pCRII (Invitrogen, Leek, The Netherlands) to yield pAH2 and transformed into *E. coli* BL21 (DE3) (21) by electroporation. After aerobic growth on M9ZB medium (21) in the presence of ampicillin (0.2 mg/ml) at 37°C, the

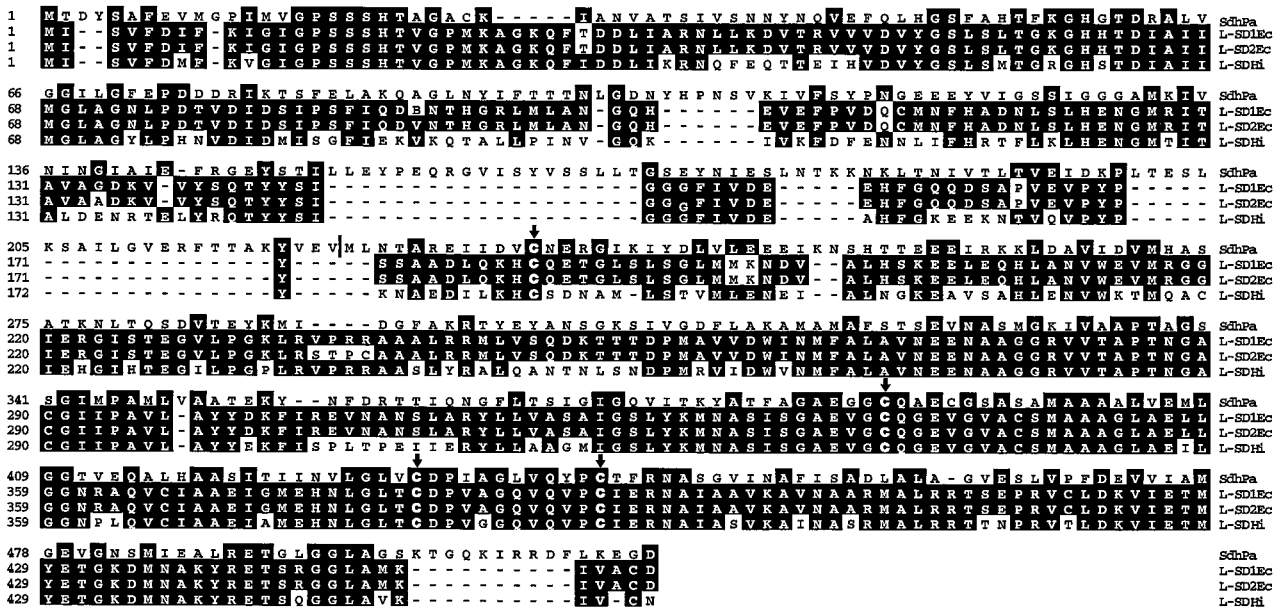

FIG. 2. Alignment of the amino acid sequences of four L-serine dehydratases by Clustal's method. The sequences of the two subunits of L-serine dehydratase from *P. asaccharolyticus* in the order β → α (SdhPa), the two one-subunit L-serine dehydratases from *E. coli* (L-SD1Ec and L-SD2Ec), and a putative L-serine dehydratase from *H. influenzae* (L-SDHi) (3) are shown. The C-terminal amino acid of SdhB is V220 and the N-terminal amino acid of SdhA is M221, as indicated by a vertical line. The four conserved cysteines are marked by arrows.

cells were incubated in M9 medium supplemented with all amino acids except methionine. Cells were labeled with 1.2 MBq of L-[^{35}S]methionine for 5 min after induction of T7 RNA polymerase with isopropylthiogalactoside (0.8 mM) and inhibition of bacterial RNA polymerase with rifampin (200 μg/ml). Crude extracts of labeled cells separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) contained two radiolabeled proteins with molecular masses of 25 and 30 kDa, as expected for the β- and α-subunits of L-serine dehydratase, respectively (Fig. 3). Scanning of the two bands by a phosphorimager showed the α-subunit to be labeled four times more brightly than the β-subunit. This result can be partially explained by the fact that the α-subunit contains three times the amount of methionines (12 methionines) as the β-subunit (4 methionines). Therefore, in *E. coli*, *sdhA* and *sdhB* are expressed in a proportion close to 1:1.

In order to facilitate purification of the enzyme, the L-serine dehydratase was overproduced in *E. coli* XL1-Blue (18) as a protein in which a His_6 tag was attached to the N terminus of the β-subunit by cloning the L-serine dehydratase genes into vector pQE30 (Qiagen, Hilden, Germany), yielding pST1. *E. coli* cells carrying pST1 were grown aerobically at 37°C in Luria-Bertani medium (18) in the presence of ampicillin (100 μg/ml) and tetracycline (15 μg/ml) to an optical density at 578 nm of 1.0, induced by 1 mM isopropylthiogalactoside for at least 1 h before harvesting. Since the enzyme is inactivated by air, all following steps were performed under an atmosphere of 95% N_2–5% H_2. Cells were sonicated in 50 mM potassium phosphate buffer, pH 8.0, containing 300 mM NaCl and centrifuged at 90,000 × *g* for 30 min. As measured by standard assay, the supernatant had a specific activity of 233 nkat/mg, which was 16-fold higher than that determined in extracts of *P. asaccharolyticus* cells (5). The His_6 affinity tag allowed the purification of the modified enzyme by affinity chromatography on Ni-NTA (nickel-nitrilotriacetate) resin according the protocol of the QIAexpress (Qiagen) system (elution of protein from the resin by a step gradient from 50 to 500 mM imidazole in 50 mM potassium phosphate, pH 7.0, containing 5 mM glycine). Unfortunately, copurification of the α-subunit, which contained no His tag, did not occur in the required 1:1 proportion. Nevertheless, an active enzyme with a specific activity comparable to that of the wild-type enzyme (His_6-SDH, 4.8 μkat/mg; wild type, 6.0 μkat/mg) (5) with a purification factor of 20 and a yield of 5% was obtained.

Fusion of *sdhB* with *sdhA* and expression in *E. coli*. Since *sdhB* and *sdhA* occur in the same reading frame, directly adjacent to each other (Fig. 1), and since the derived amino acid sequence of both genes together shows significant similarity to L-SD1 and L-SD2 from *E. coli*, each of which consists of only one subunit, we wanted to investigate the effect of a fusion of

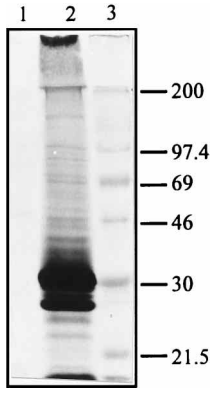

FIG. 3. Autoradiogram after expression of *sdhBA* in *E. coli* in the presence of [^{35}S]methionine. Lane 1, *E. coli* harboring the "empty" vector (negative control); lane 2, *E. coli* harboring the vector with the two structural genes of the L-serine dehydratase (both subunits [α = 30 kDa, β = 25 kDa] can be detected as labeled protein bands); lane 3, marker proteins with sizes in kDa.

sdhB to *sdhA*. The stop codon (TAG) was thus changed to a glycine codon (GGT) by site-directed mutagenesis following the protocol of the Sculptor in vitro mutagenesis system (Amersham, Braunschweig, Germany). The resulting fusion protein was overproduced in *E. coli* XL1-Blue (18) carrying six histidine residues at the N terminus. Under denaturing purification conditions, according to the protocol of the QIAexpress system, the fusion protein was detected by SDS-PAGE. Anaerobic extracts of aerobically grown *E. coli* cells harboring the *sdhBA*-containing plasmid with the mutation (pST2) had a specific L-serine dehydratase activity of 233 nkat/mg. The purification of the His_6 fusion protein under nondenaturing conditions was not as successful as that of the His_6-L-serine dehydratase, probably because of weak binding to the Ni-NTA resin. It was purified up to a specific activity of 1.8 μkat/mg, but clear detection of the protein by SDS-PAGE was not possible. Like the β-His_6-L-serine dehydratase, the His_6 fusion protein was inactivated by air. The half-life observed by air inactivation of the fusion protein (2 to 3 h) was somewhat shorter than that of the β-His_6-protein and of the wild type enzyme (6 h) (5). Both modified L-serine dehydratases were stable under anaerobic conditions for at least 10 h.

Functional complementation of an *E. coli* mutant deficient in L-SD1. In order to examine whether the [4Fe-4S] cluster containing L-serine dehydratase from *P. asaccharolyticus* can functionally replace one of the corresponding enzymes from *E. coli* under physiological conditions, an expression vector carrying *sdhBA* (pST1) was introduced into *E. coli* MEW22 (22). This strain is deficient in L-SD1 and can be distinguished from the parent strain MEW1 (14) by its inability to grow on a minimal medium with L-serine, glycine, and L-leucine as the major carbon sources (SGL medium) (22). Synthesis of β-His_6-L-serine dehydratase from *P. asaccharolyticus* in MEW22 restored the mutant's ability to form visible colonies on solid SGL medium within 4 days, whereas MEW22 cells harboring the plasmid that lacked *sdhBA* did not grow in the same period. Nevertheless, the growth rate of the complemented MEW22 was lower than that of its parent strain, MEW1, which formed visible colonies on SGL medium within 1 day. To confirm that complementation was not due to MEW22 mutations, the plasmid carrying *sdhBA* was isolated and retransformed in MEW22. Growth studies with these retransformants showed the same results.

Discussion. We demonstrated that the [4Fe-4S] cluster containing L-serine dehydratase from *P. asaccharolyticus* is structurally and functionally related to the two L-serine dehydratases, L-SD1 and L-SD2, present in *E. coli*. Furthermore, L-serine dehydratase from *P. asaccharolyticus* was shown to complement an *E. coli* mutant deficient for L-SD1 in its ability to grow on a minimal medium containing L-serine, glycine, and L-leucine as the only major carbon sources.

The α-subunit of L-serine dehydratase from *P. asaccharolyticus* contains four conserved cysteine residues, three of which may be involved in coordinating three irons of the [4Fe-4S] cluster. Interestingly, the conserved cysteine residues followed spacings found neither in the ferredoxins nor in aconitase (23). This finding was unexpected, since previous electron paramagnetic resonance spectroscopic investigations of L-serine dehydratase from *P. asaccharolyticus* had suggested a mechanism of catalysis similar to the one elucidated for aconitase (8).

The structural genes for the α- and β-subunits of L-serine dehydratase from *P. asaccharolyticus* occur in the same reading frame, directly adjacent to each other. Together with the sequence homology with L-SD1 and L-SD2 from *E. coli*, both of which consist of only one subunit, this finding suggested that the single subunit of the enzymes from *E. coli* might have resulted from a gene fusion during the course of evolution. In support of this view, an active recombinant fusion protein of L-serine dehydratase from *P. asaccharolyticus* was created.

The iron-sulfur-dependent mechanism of deamination of L-serine has long escaped recognition due to the unexpected oxygen lability of the involved enzymes. The described sequence similarities between the enterobacterial and clostridial enzymes support our earlier speculation (6) that in Bacteria, L-serine is dehydrated in general by the aid of an iron-sulfur cluster, whereas L-threonine requires pyridoxal-5′-phosphate for its deamination to 2-oxobutyrate in an otherwise completely analogous reaction. The question of whether the two different mechanisms evolved just by chance or as a result of a chemical necessity arises (13). We favor the latter explanation since, due to the hyperconjugative effect of the methyl group, the hydroxyl group is much more easily eliminated from threonine than from serine. Hence, in order to get the most efficient enzymes in serine, the hydroxyl group has to be activated, whereas lowering the pK of the α-hydrogen in threonine by forming a Schiff's base with pyridoxal-5′-phosphate may optimize the catalytic power of L-threonine dehydratases. In mammals, in which L-serine also is deaminated by pyridoxal-5′-phosphate-dependent enzymes, several iron-sulfur-dependent dehydratases (e.g., the two extant iron-sulfur-containing fumarases found in *E. coli*) (4) have apparently been abandoned during evolution, due probably to their oxygen sensitivity. An apparent exception to these considerations is represented by the pyridoxal-5′-phosphate-dependent D-serine dehydratase from *E. coli*. The function of this enzyme, however, has been attributed to detoxification rather than to a rate-limiting step in energy metabolism (11).

Nucleotide sequence accession number. The nucleotide sequence for *sdhA* and *sdhB* has been deposited with GenBank under accession no. U76260.

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