A novel lysine 2,3-aminomutase encoded by the *yodO* gene of *Bacillus subtilis*: characterization and the observation of organic radical intermediates

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The *yodO* gene product of *Bacillus subtilis* has been cloned and overexpressed in *Escherichia coli* and purified. The nucleotide sequence encodes a protein of 471 amino acids with a calculated molecular mass of 54071 Da. The translated amino acid sequence is more than 60 % identical to that of the lysine 2,3-aminomutase from *Clostridium subterminale* SB4. Analytical HPLC gel-permeation chromatography leads to an estimate of an overall molecular mass of 224000 ± 21000 Da, which corresponds to a tetrameric protein. The purified protein contains iron, sulphide and pyridoxal 5′-phosphate (PLP) and displays an optical absorption band extending to 700 nm, suggesting the presence of an iron-sulphide cluster. After reductive incubation with L-cysteine anaerobically, the protein catalyses the transformation of L-lysine into β-lysine in the presence of *S*-adenosylmethionine (AdoMet) and sodium dithionite. The K_m value for L-lysine is estimated to be 8.0 ± 2.2 mM. The iron-sulphur centre is stable in air, allowing aerobic purification. EPR spectroscopy at 10 K of the purified enzyme revealed an EPR signal similar to that of the $[4Fe\text{-}4S]^{3+}$ cluster observed in the clostridial lysine 2,3-aminomutase. Incubation with cysteine under anaerobic conditions converts the iron-sulphur centre into the EPR-silent $[4Fe\text{-}4S]^{2+}$. Unlike the clostridial enzyme, the fully reduced $[4Fe\text{-}4S]^{+}$ could not be characterized by further reduction with dithionite in the presence of AdoMet, although both dithionite and AdoMet were required to activate the enzyme. Upon addition of L-lysine, dithionite and AdoMet to the reduced enzyme and freezing the solution to 77 K, the EPR spectrum revealed the presence of an organic free-radical signal ($g = 2.0023$), which displayed multiple hyperfine transitions very similar to the spectrum of the β-lysine-related radical in the mechanism of the clostridial lysine 2,3-aminomutase. Experiments with isotopically substituted L-lysine and lysine analogues verified the association of spin density with the carbon skeleton of lysine. The data indicate that the protein encoded by the *yodO* gene of *B. subtilis* is a novel lysine 2,3-aminomutase. The *E. coli* homologue of clostridial lysine 2,3-aminomutase was also expressed in *E. coli* and purified. This protein contained iron and sulphide but not PLP, it did not display lysine 2,3-aminomutase activity, and addition of PLP did not induce 2,3-aminomutase activity.

Key words: *S*-adenosylmethionine, cysteine, EPR spectroscopy, iron-sulphur cluster, pyridoxal phosphate.

INTRODUCTION

Lysine 2,3-aminomutase catalyses the interconversion of L-lysine and L-β-lysine, which involves migration of the α-amino group to the β-carbon with the concomitant transfer of the β-hydrogen to the α-carbon. This enzyme plays a role in the utilization of lysine for growth of the anaerobe *Clostridium subterminale* SB4 [1–3]. β-Lysine is also a precursor in the biosynthesis of antibiotics, such as viomycin and streptothricin, in *Streptomyces* [4,5]. Therefore, enzymes that convert α-amino acids into β-amino acids are potentially useful for production in the pharmaceutical industry. Lysine 2,3-aminomutase from *C. subterminale* SB4 is the only well-characterized amino acid 2,3-aminomutase [6–8].

The reaction catalysed by lysine 2,3-aminomutase is analogous to the adenosylcobalamin-dependent rearrangements, in which hydrogen transfer is mediated by the adenosyl moiety of the coenzyme. Unlike adenosylcobalamin-dependent enzymes, clostridial lysine 2,3-aminomutase is not adenosylcobalamin-dependent, but contains [4Fe-4S] clusters and pyridoxal 5′-phosphate (PLP) and is activated by *S*-adenosylmethionine (AdoMet). AdoMet and [4Fe-4S] clusters are thought to function in place of adenosylcobalamin in providing for the transient and reversible formation of the 5′-deoxyadenosyl radical. A radical rearrangement mechanism has been proposed for the clostridial enzyme [9–11], in which the 5′-deoxyadenosyl radical initiates the rearrangement by abstracting a hydrogen atom from C-3 of L-lysine to form the substrate-based free radical **1** (Scheme 1). PLP facilitates the rearrangement by forming an aldimine linkage to the migrating nitrogen [12,13] and allowing the transformation of radical **1** into the product-related radical **3**, presumably through the intermediate formation of **2**.

Based on the amino acid sequence of lysine 2,3-aminomutase from *C. subterminale* SB4, seven homologous proteins have been identified in the prokaryotic database [14]. The amino acid sequences of these proteins share 32–72 % identities with *C. subterminale* lysine 2,3-aminomutase. The *Bacillus subtilis* protein encoded by the *yodO* gene [15] is 62 % identical, and the *Escherichia coli* protein is 32 % identical. A prominence that is similar in all of these proteins is the cysteine motif that is thought to be the iron binding site. This is shown in Figure 1(A), in which appears the striking and unique presence of at least three conserved arginine residues in all eight motifs. The sequences are shown top to bottom in order of decreasing overall identity to *C. subterminale* lysine 2,3-aminomutase. The eight homologous proteins may be differentiated by their ability to bind PLP, as suggested in Figure 1(B). The upper group of four, including the *B. subtilis* protein, incorporate a putative PLP-binding motif,

Abbreviations: PLP, pyridoxal 5′-phosphate; AdoMet, *S*-adenosylmethionine; AdoHCys, *S*-adenosylhomocysteine; IPTG, isopropyl β-D-thiogalactoside; DTT, dithiothreitol.

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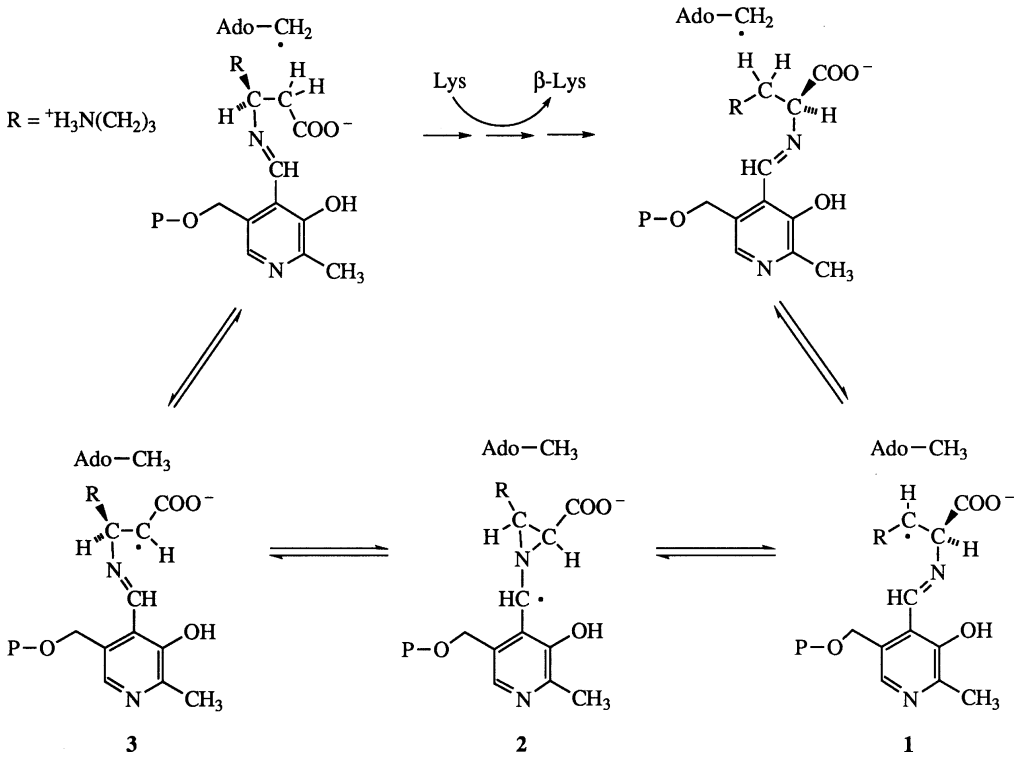
Scheme 1



Scheme 1 The proposed mechanism of clostridial lysine 2,3-aminomutase

which is similar to that associated with serine and threonine dehydratases. The lower group of five homologues, including the *E. coli* protein, lacks this motif.

Here we report the identification of the protein encoded by the *yodO* gene of *B. subtilis* as a lysine 2,3-aminomutase, as well as the characterization of this new member of the lysine aminomutase family. Like the clostridial enzyme, but unlike adenosylcobalamin-dependent enzymes, the *B. subtilis* lysine 2,3-aminomutase contains iron, inorganic sulphide and PLP and is activated by AdoMet. Similarities with the clostridial enzyme notwithstanding, the *B. subtilis* lysine 2,3-aminomutase exhibits distinctive physical, chemical and mechanistic properties. Of particular interest is its remarkable stability in air, which facilitates mechanistic studies and makes it potentially useful for applications in the synthesis of pharmaceutical agents with antibiotic properties. We also show that the homologous protein from *E. coli* lacks PLP and is not a lysine 2,3-aminomutase.

MATERIALS AND METHODS

Materials

B. subtilis strain A.T.C.C. 27505 was obtained from the American Type Culture Collection. L-[3,3,4,4,5,5,6,6-$^2H_8$]Lysine (99% enrichment) was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). AdoMet, *S*-adenosylhomocysteine (AdoHCys), PLP and 4-thia-L-lysine were from Sigma. AdoMet was purified by chromatography using a 2 cm × 20 cm column of CM-cellulose before use. All other compounds were used directly without further purification.

Cloning of *B. subtilis yodO*

The *yodO* gene was isolated from *B. subtilis* chromosomal DNA by PCR. Primers were prepared that produced a 1.4-kb insert containing *Nde*I and *Xho*I restriction sites necessary for cloning into pET 23a(+) plasmid vector under the control of the T7 promoter. The PCR primers used were: (+) strand, 5′-TATACATATGAAAAACAAATGGTATAAACCGAAACGGCATTGGAAGG-3′; and (−) strand, 5′-TAGACTCGAGTCATGAAGAATCCCCTCCGCATTCAGTCTCTTTCTG-3′. Chromosomal DNA from *B. subtilis* (Ehrenberg) Cohn (A.T.C.C. 15818) was prepared using the Qiagen Genomic Tip 500/G from cells grown in nutrient broth (Difco #3). The PCR reaction mixture (100 μl total volume) contained: *B. subtilis* chromosomal DNA, 0.5 μg; *Pfu* DNA polymerase reaction buffer (Stratagene, La Jolla, CA, U.S.A.); dNTPs, 0.2 mM each; oligonucleotide primers, 0.2 μM each; and Turbo *Pfu* DNA polymerase, 5 units. Samples were subjected to 30 cycles of 1 min at 96 °C, 1 min at 60 °C and 3 min at 72 °C. After thermocycling, DNA formed during the PCR process was purified by 2% agarose gel electrophoresis in 0.04 M Tris/acetate, pH 8.0, with 1 mM EDTA. Following identification and excision of the appropriately sized (≈ 1.4 kb) ethidium bromide-stained band, DNA was extracted from the agarose, ethanol-precipitated and resuspended in 0.01 M Tris/HCl/EDTA (TE) buffer, pH 8. The purified PCR product was blunt-end ligated to pCR-Script Amp cloning vector (Stratagene) using 0.3 pmol of insert/0.005 pmol of vector according to the manufacturer's specification. The ligated DNA was used to transform XL1-MRF′ *E. coli* cells, which were subsequently plated on Luria–Bertani/carbenicillin (100 μg/ml)/

A.

138	**C**SMY**C**RH**C**TRRRF 150	*C. subterminale* KAM
	CSMY**C**RH**C**TRRRF	*P. gingivalis*
134	**C**SMY**C**RY**C**TRRRF 146	*B. subtilis* KAM
	CASY**C**RY**C**TRSRI	*D. radiodurans*
	CAVY**C**RH**C**MRKRI	*A. aeolicus*
	CFSH**C**RY**C**FRRGF	*T. pallidum*
	CAVN**C**RY**C**FRRHF	*H. influenzae*
	CAVN**C**RY**C**FRRHF	*E. coli*

B.

293	**LVKIRV** 298	*C. subterminale* KAM
	LVKMRV	*P. gingivalis*
288	**LVKIRV** 293	*B. subtilis* KAM
	LVKIRV	*D. radiodurans*
	LLRIKV	*A. aeolicus*
	LTCLGV	*T. pallidum*
	LFQTGI	*H. influenzae*
	LFDAGV	*E. coli*
	SFKIRG	Rat liver serine dehydratase
	SFKLRG	Yeast threonine dehydratase
	SFKIRG	*E. coli* L-threonine dehydratase
	SIKARG	*E. coli* D-threonine dehydratase

Figure 1 Amino acid sequences of iron- and PLP-binding motifs in clostridial lysine 2,3-aminomutase and its homologues

(**A**) Iron-binding motifs of *C. subterminale* lysine 2,3-aminomutase and its homologues. The cysteine residues that are proposed to bind the iron sulphide cluster are shown in bold. Note the presence of at least three conserved arginine residues. (**B**) Putative PLP-binding motif of *C. subterminale* lysine 2,3-aminomutase and the corresponding sequences in its homologues. Note that the upper four homologous proteins, including the *B. subtilis* protein, retain the KXR motif of the PLP-binding sites in four serine and threonine dehydratases, which are also shown. This motif is absent in the lower five homologous proteins, including that from *E. coli*. *P. gingivalis*, *Porphyromonas gingivalis*; *D. radiodurans*, *Deinococcus radiodurans*; *A. aeolicus*, *Aquifex aeolicus*; *T. pallidum*, *Treponema pallidum*; *H. influenzae*, *Haemophilus influenzae*.

isopropyl β-D-thiogalactoside (IPTG)/X-Gal plates and cultured overnight. White colonies were chosen for subcloning into pET23a(+).

Plasmid DNA (10 μg) was double-digested with *Nde*I/*Xho*I, and the *yodO* fragment gel-purified as before. pET-23a(+) (10 μg) was similarly digested with *Nde*I/*Xho*I, dephosphorylated with 1 unit of calf-intestine alkaline phosphatase for 30 min at 37 °C, gel-purified and ethanol precipitated. *yodO* and cut pET-23a(+) were ligated with T4 DNA ligase overnight at 14 °C. Competent *E. coli* cells (DH5α, Life Technologies) were transformed and cells plated on Luria–Bertani/carbenicillin plates. Plasmid DNAs from individual colonies were sequenced to confirm the construct. For expression of *B. subtilis yodO* gene in *E. coli*, pAF-98/*yodO* was transformed into competent BL21(DE3) *E. coli* cells (Novagen). For transformation, 20 ml of competent cells were transformed with 0.1 μg of plasmid DNA. The sequence was verified in its entirety by DNA sequencing using the automated ABI Prism Dye Terminator Cycle Sequencing procedure (University of Wisconsin Biotechnology Center, Madison, WI, U.S.A.).

Cloning of *E. coli* unknown gene (P39280)

The *E. coli* gene P39280 of unknown function was isolated from *E. coli* chromosomal DNA (JM109) by PCR according to the procedure described above. The PCR primers used were: (+) strand, 5′-ATGGCGCATATTGTAACCCTAAATAC-3′, and (−) strand, 5′-TTACTGCTGGCGTAGCTGGAGATC-3′. DNA from PCR was cloned into pET-23a(+) vector at the *Nde*I/*Xho*I restriction sites. The final plasmid construct was transformed into *E. coli* BLR(DE3) plysS cells (Novagen) for protein expression.

Expression of recombinant lysine 2,3-aminomutase (*yodO*) and unidentified *E. coli* gene

E. coli strain BL21(DE3) carrying the expression vector pAF-98/*yodO* was grown aerobically at 37 °C in shake flasks containing Terrific Broth medium plus 100 μg/ml ampicillin and 0.5 mM IPTG. High-level expression of the active enzyme was achieved when cells were grown into the stationary phase. The unknown *E. coli* gene product was expressed in cells grown anaerobically in a 15-l fermentor following sparging with nitrogen. The cells were cultured in 2 × YT medium (16 g/l bactotryptone, 10 g/l bacto-yeast extract and 5 g/l NaCl in distilled water) supplemented with 50 mM $FeSO_4$, 50 mM sodium sulphide, 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. When the cell density had reached D_{600} of 0.6, IPTG (1 mM) was added and the culture continued for an additional 3 h. In both cases, cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −70 °C.

Protein purification

In the purification of the recombinant *B. subtilis yodO* gene product (lysine 2,3-aminomutase), all steps were carried out at 4 °C unless otherwise stated. The harvested cells were lysed by sonication. Cell debris, large ribosomal protein and RNA were removed by ultracentrifugation for 45 min at 46000 revs./min (174000 ***g***) using a Beckman 55.2Ti rotor. Streptomycin sulphate was added slowly to the supernatant to a concentration of 0.1 %, and precipitated nucleic acids were removed by centrifugation at 39000 ***g***. Ammonium sulphate was added slowly to 40 % saturation, the precipitated protein was removed by centrifugation, ammonium sulphate was added to 55 % saturation, and the suspension was centrifuged at 27000 ***g***. The pellet was redissolved in a minimum volume of 100 mM Hepps buffer at pH 8.0 containing 50 μM lysine and 25 μM PLP, loaded on to a 120 cm × 2.5 cm Sephacryl S-300 column (Pharmacia), and eluted with the same buffer containing 20 mM NaCl. Fractions displaying absorbance at 410 nm were pooled and loaded on to a 2.5 cm × 30 cm DEAE-Sephacel column (Pharmacia), which was eluted with a linear gradient formed from 1 l of the Sephacryl elution buffer and 1 l of the same buffer containing 500 mM NaCl. Fractions displaying absorbance at 410 nm were pooled and concentrated using an Amicon concentrator. The purified enzyme was stored at −70 °C after being frozen in liquid nitrogen. Anaerobic purifications were carried out at room temperature in a Coy anaerobic chamber except for preparative centrifugations, in which centrifuge bottles filled and sealed in the anaerobic chamber were removed for centrifugation and later returned to the chamber before being opened.

The recombinant gene product from the expression of the *E. coli* unknown gene was purified by methods described previously for the purification of clostridial lysine 2,3-aminomutase [16,17].

Protein and cofactor analysis

The purity of *Bacillus* lysine 2,3-aminomutase was judged by inspection of Coomassie Brilliant Blue-stained gels from SDS/PAGE and the chromatogram of analytical HPLC gel filtration of the protein. Routine measurements of enzyme concentrations were performed using the BCA protein assay reagent (Pierce). The N-terminal amino acid sequence of the purified protein was

determined at the Macromolecular Structure Facility of Michigan State University (East Lansing, MI, U.S.A.). The subunit molecular mass was measured by electrospray ionization MS to an accuracy of $\pm 0.02\%$ at the Biotechnology Center of the University of Wisconsin-Madison.

Metal content of the purified enzyme was analysed by inductively coupled plasma-emission spectroscopy (Soil Science Lab, University of Wisconsin-Madison). The total enzyme-bound PLP was measured spectrophotometrically for solutions of known enzyme concentrations using the phenylhydrazine method [18]. Analyses for inorganic sulphide was carried out by the method of Beinert [19].

Enzyme activation and assay

Enzyme was pre-incubated in 100 mM Hepps buffer at pH 8.0 with 8.0 mM L-cysteine in the presence or absence of 0.5 mM PLP and/or 1.0 mM ferrous ammonium sulphate at 37 °C for 3 h in a Coy anaerobic chamber before assaying for activity. Enzyme activity was assayed according to the procedure of Chirpich et al. [2] as modified by Ballinger et al. [6], in which 1 unit of activity was defined as the amount of enzyme required to produce 1.0 μmol of β-lysine$\cdot$min^{-1} under standard conditions. Owing to its low activity relative to the clostridial lysine 2,3-aminomutase, the activity of the *Bacillus* enzyme is expressed in m-units. In a typical radiochemical assay, the reductively pre-incubated lysine 2,3-aminomutase was further diluted before mixing with L-[U-^{14}C]lysine, sodium dithionite and AdoMet to give final concentrations of 44 mM L-[U-^{14}C]lysine, 3.5 mM dithionite and 80 μM AdoMet in 100 mM Hepps buffer at pH 8.0. When PLP and/or Fe(II) were included in the pre-activation mixture, the final concentrations of PLP and Fe(II) in the assay mixtures were 45 and 90 μM, respectively. Reactions were quenched at different time intervals with 0.5 M formic acid. Radiolabelled α- and β-lysine were separated by paper electrophoresis, and the radioactivity was measured by scintillation counting as described in [2,6]. HPLC assays of the reaction were carried out by derivatizing an aliquot of the reaction mixture with phenylisothiocyanate before chromatography and comparison with standards as described in [20,21].

Steady-state kinetic parameters of the recombinant lysine 2,3-aminomutase from *Bacillus* were estimated after reductive incubation in the presence of cysteine, PLP and ferrous ammonium sulphate. Initial rates for the conversion of lysine into β-lysine were measured at 37 °C under the conditions described above for assaying the enzyme, except that the concentration of lysine was varied from 2.4 to 48 mM. Kinetic constants and their associated errors were determined by fitting rate data to the Michaelis–Menten equation using the weighted linear-regression analysis described by Wilkinson [30].

EPR spectroscopy

In preparation of EPR samples, the enzyme was incubated (4:1) with the activation buffer, consisting of 200 mM Hepps buffer at pH 8.0, 40 mM cysteine, 2.5 mM PLP and 5.0 mM ammonium iron (II) sulphate at 37 °C for 3 h. Enzyme concentrations in the activation mixture for EPR samples are ≈ 200 μM. For a multiple-sample experiment, a reductively pre-incubated enzyme mixture was prepared for all samples. For each sample, an aliquot of reductively pre-incubated enzyme was mixed with Hepps buffer at pH 8.0, substrate, dithionite and AdoMet in a microcentrifuge tube to give a final volume of 250 μl and transferred to a 3-mm (inner diameter) quartz EPR tube. The concentrations of Hepps, lysine, dithionite and AdoMet in a typical EPR sample were 200 mM, 67.2 mM, 2.5 mM and 2.0 mM, respectively. Components excluded in controls were replaced with 200 mM Hepps buffer (pH 8.0). The EPR sample tubes were then frozen in liquid nitrogen within 4–5 min. For studying the correlation of enzyme activity and spin concentrations, ^{14}C-labelled L-lysine was used and an aliquot was withdrawn for radiochemical assays before the sample was frozen in the EPR tube.

EPR spectra were recorded at X-band on a Varian E3 spectrometer. A standard liquid nitrogen immersion dewar was used. Low-temperature X-band EPR spectra were acquired on a Varian E Line spectrometer equipped with a Varian E102 microwave bridge. An Oxford Instruments ESR-900 continuous-flow helium cryostat Oxford Instruments 3120 temperature controller were used. Both the spectrometers were connected with an IBM AT microcomputer for data acquisition and analysis. Spin concentrations were determined by integration of spectra, using a Cu-EDTA standard as a reference [22]. All spectra were obtained as averages of three 4-min scans.

RESULTS

Characterization of the *yodO* gene product

By making use of two primers complementary to the nucleotide sequences at the 5′ and 3′ termini of the *yodO* gene, a 1.4-kb DNA fragment was amplified by PCR from the genomic *Bacillus* DNA and cloned into an expression vector pET-23a(+). A base change was made in the upstream primer so that the leucine, encoded by the first codon of the open reading frame, was changed to methionine. The cloned DNA fragment encodes a peptide of 471 amino acid residues with a calculated molecular mass of 54071 Da. The amino acid sequence predicted from the cloned DNA displays 62% sequence identity to that of the clostridial lysine 2,3-aminomutase.

The protein was expressed in *E. coli* and purified to apparent homogeneity, as judged by SDS/PAGE and HPLC molecular-filtration chromatography. The purification steps and associated data on yield and purity are set forth in Table 1. N-terminal amino acid sequence analysis generated the sequence MKNK-WYKPKR, which confirmed that the purified protein is the *yodO* gene product. As shown in Figure 1(A), the sequences contain three conserved cysteine residues, a motif for metal ligands often found in iron-sulphur-cluster-containing enzymes [23]. Also of interest is a putative consensus sequence for PLP binding found in some PLP-containing proteins [24] (Figure 1B).

Calibrated molecular-filtration analysis of protein samples under non-denaturing conditions led to an estimated molecular mass of 224000 ± 21000 Da. The purified protein could be dissolved in 90% formic acid to disrupt non-covalent interactions, and subsequent analysis by electrospray ionization MS gave a molecular mass of 54061 ± 10 Da. The value matches the calculated mass of 54071 Da based on the nucleotide sequence of the gene. The gel-permeation and mass-spectrometric results suggest a homotetrameric quaternary structure for the protein, in contrast to the hexameric structure of the clostridial enzyme.

The dashed line in Figure 2 is the UV-visible absorption spectrum of the purified protein. The spectrum consists of the protein band at 280 nm, a band at 420 nm and a long-wavelength tail extending to 700 nm. The 420-nm band is attributed mainly to the iron-sulphur centre, although PLP may make a minor contribution at this wavelength. A similar 420-nm band is observed in the homologous, PLP-free *E. coli* protein, which is discussed in a later section.

The *yodO* gene product was analysed for transition metal ions, inorganic sulphide and PLP, with the results shown in Table 2. The *B. subtilis* yodO protein contains iron, inorganic sulphide

Table 1 Purification of recombinant *Bacillus* lysine 2,3-aminomutase

Details of the purification procedure are given in the Materials and methods section.

Step	Total protein (mg)	Total activity (m-units)	Specific activity (m-units · mg^{-1})	Yield (%)	Purification (fold)
Streptomycin fraction	1850	184	99	100	1.0
$(NH_4)_2SO_4$ fraction	690	153	222	83	2.2
Sephacryl S-300	338	107	317	58	3.2
DEAE-Sephacel	109	68	623	37	6.3

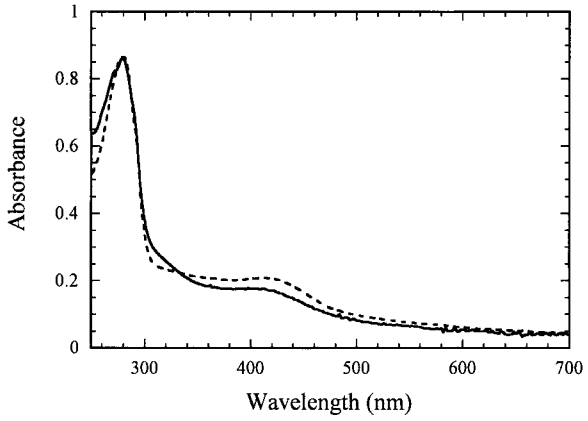


Figure 2 UV-visible absorption spectra of clostridial lysine 2,3-aminomutase homologues

Dashed line: the spectrum of the *B. subtilis* protein obtained in 50 mM Hepps buffer at pH 8.0 in the presence of 1 mM DTT and 0.05 mM lysine. Solid line: the spectrum of the *E. coli* P39280 gene product. Note that both the *B. subtilis* and *E. coli* proteins display the 420-nm band and the long-wavelength tail characteristic of iron sulphide proteins.

and PLP. The sulphide and iron are stoichiometrically comparable and correspond to ≈ 8 Fe and 8 S^{2-} per tetramer, and the PLP analysis indicates four PLP per tetramer. The data are consistent with the presence of either two [4Fe-4S] clusters or four [2Fe-2S] clusters per tetramer.

Biochemical activity of the *yodO* gene product

The cofactor content and high degree of identity in primary structure with the clostridial lysine 2,3-aminomutase suggest that the *yodO* protein is an amino acid aminomutase. Such activity was sought using L-arginine, L-ornithine, L-phenylalanine, L-leucine and L-isoleucine as substrates in addition to L-lysine. Under assay conditions similar to those used for the clostridial lysine 2,3-aminomutase [6], we found the yodO protein to be a lysine 2,3-aminomutase, and none of the other amino acids were accepted as substrates. Like the clostridial enzyme, a pre-incubation process is required to activate the enzyme. However, unlike the clostridial enzyme, which displays full activity after reductive pre-incubation with dihydrolipoate or dithiothreitol (DTT) in the presence of Fe(II), the *B. subtilis* enzyme requires the presence of L-cysteine instead of dihydrolipoate or DTT to achieve maximum activity, and added Fe(II) is not necessary. Table 3 summarizes various conditions used to activate the enzyme and the activities measured in each case. The complete reductive incubation mixture contained 8.0 mM cysteine, 1.0 mM

Table 3 Effect of various reductive incubation mixtures on lysine 2,3-aminomutase activity

Reductive incubation was in 50 mM Hepps buffer at pH 8.0 and at 37 °C for 3 h in a Coy anaerobic chamber. Concentrations of reactants were: cysteine, 8 mM; Fe(II), 1.0 mM; PLP, 0.5 mM; DTT, 35 mM; mercaptoethanol, 70 mM; dihydrolipoate, 20 mM; and lysine 2,3-aminomutase, 20–30 μM. Activities were measured in 0.2 M Hepps buffer at pH 8.0 with 44 mM L-lysine, 3.5 mM dithionite and 35 μM AdoMet at 37 °C; means ± S.D. are shown.

Experiment	Reductive incubation mix	Activity measured (m-units · mg^{-1})
1	Complete [cysteine, Fe(II), PLP]	660 ± 51
2	− Reductive incubation	38 ± 6
3	− Fe(II)	640 ± 58
4	− PLP	580 ± 34
5	− Cysteine	215 ± 15
6	− Cysteine, + DTT	190 ± 17
7	− Cysteine, + mercaptoethanol	210 ± 9
8	− Cysteine, + dihydrolipoate	177 ± 12
9	Complete + DTT	417 ± 20

Table 2 Transition-metal ions, sulphide and PLP in *Bacillus* lysine 2,3-aminomutase (in mol per mol of tetrameric enzyme)

Iron was measured by inductively coupled plasma-emission spectroscopy, sulphide was measured by the method of Beinert [19], and PLP was measured by the phenylhydrazine method of Wada and Snell [18]. Means ± S.D. are shown.

Ion	Enzyme preparation… A	B	C	D
Iron	6.64 ± 0.74	7.67 ± 0.61	8.40 ± 0.87	–
Cobalt	0.12 ± 0.04	0.05 ± 0.04	0.03 ± 0.03	–
Copper	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	–
Zinc	1.78 ± 0.09	0.98 ± 0.07	0.77 ± 0.08	–
Sulphide	–	–	7.31 ± 0.56	8.41 ± 0.62
PLP	–	3.60 ± 0.25	3.87 ± 0.31	4.20 ± 0.32

Table 4 Effect of AdoMet, L-methionine and 5′-deoxyadenosine on activity

Enzyme was reductively incubated in 50 mM Hepps buffer with 8 mM L-cysteine at pH 8.0 and 37 °C for 3 h inside a Coy anaerobic chamber. Concentrations of components were, where applicable: enzyme, 1.5–2.0 μM; lysine, 44 mM, dithionite, 3.5 mM; AdoMet, 80 μM; L-methionine, 3.6 mM; and 5′-deoxyadenosine, 3.6 mM. Means ± S.D. are shown.

Experiment	Reaction mixture	Activity measured (m-units · mg^{-1})
1	Complete (activated enzyme, dithionite, AdoMet)	620 ± 50
2	+ L-Methionine	600 ± 73
3	+ 5′-Deoxyadenosine	450 ± 24
4	+ L-Methionine + 5′-deoxyadenosine	420 ± 18
5	− AdoMet	4.9 ± 0.4
6	− AdoMet, + L-methionine	3.5 ± 0.3
7	− AdoMet, + 5′-deoxyadenosine	7.9 ± 0.3
8	− AdoMet, + L-methionine + 5′-deoxyadenosine	8.2 ± 0.4

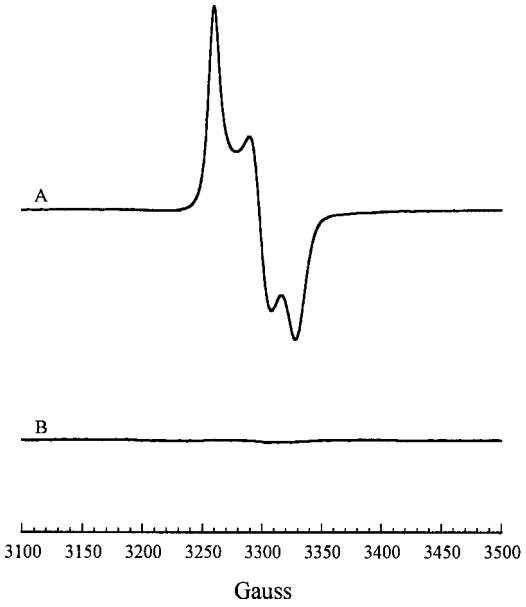


Figure 3 Low-temperature X-band EPR spectra of *B. subtilis* lysine 2,3-aminomutase

Spectra were acquired at 10 K and normalized to 60 mM aminomutase (tetramer). Spectra were recorded at 9.24 GHz, with modulation amplitude of 5 G at 1 mW power. The receiver gain was 2.5×10^3. (**A**) Spectrum of the purified enzyme in 100 mM Hepps buffer at pH 8.0. The specific activity of the enzyme was 520 m-units · mg^{-1}. (**B**) Spectrum of activated enzyme. The enzyme was activated by incubation in 50 mM Hepps buffer at pH 8.0 with 8.0 mM cysteine, 1.0 mM Fe (II) and 0.5 mM PLP in an anaerobic chamber for 4 h at 37 °C. The receiver gain was 2.5×10^3.

Fe(II) and 0.5 mM PLP. Incubation of the purified enzyme in this mixture increases the activity more than 17-fold (Table 3, experiment 1), as compared with the enzyme sample assayed in the absence of reductive incubation (Table 3, experiment 2). Omitting Fe(II) or PLP in the pre-activation has little or no effect on activity (Table 3, experiments 3 and 4), showing that the iron and PLP intrinsic to the protein (Table 2) correspond to the full complement of these cofactors. Omitting cysteine from the activation mixture leads to dramatically less activity (Table 3, experiment 5). DTT, 2-mercaptoethanol or dihydrolipoate cannot substitute for cysteine (Table 3, experiments 6–8). In fact, DTT is inhibitory when cysteine is present (Table 3, experiment 9).

The reductively pre-incubated enzyme catalyses the conversion of L-lysine into β-lysine only when sodium dithionite and AdoMet are added. Omission of either dithionite or AdoMet leads to a very low residual activity. The apparent K_m value for L-lysine was estimated to be 8.0 ± 2.2 mM.

The specific activity of a typical enzyme preparation was ≈ 620 ± 50 m-units · mg^{-1} when the standard pre-activation process (Table 3, exp. 1) was used. This activity is about 1.7 % that of the clostridial enzyme [11]. Enzyme samples purified under anaerobic conditions exhibited similar activities, and an anaerobically purified enzyme prepared from anaerobically grown *E. coli* cells also failed to display a higher activity. Unlike the clostridial enzyme, which is extremely oxygen-sensitive, the *B. subtilis* enzyme purified aerobically displays full activity after reductive pre-incubation, indicating a remarkable stability of the enzyme in air.

Effects of L-methionine and 5′-deoxyadenosine on activity

As discussed above, the reductively pre-incubated enzyme requires AdoMet and dithionite for activity (Table 4, experiment 1). In the case of the clostridial enzyme, dithionite reduces the iron-sulphur cluster [11], and AdoMet participates directly in hydrogen transfer [12]. AdoMet is transiently cleaved to L-methionine and 5′-deoxyadenosine in the course of catalysis [12]. Under the complete assay conditions, the presence of L-methionine in the assay mixture does not affect enzyme activity (Table 4, experiment 2). However, added 5′-deoxyadenosine significantly inhibits the enzyme (Table 4, experiment 3). This inhibition is presumably caused by competitive binding of 5′-deoxyadenosine to the AdoMet-binding site. The presence of both Met and 5′-deoxyadenosine causes the same inhibition as 5′-deoxyadenosine alone (Table 4, experiment 4).

When assayed in the absence of added AdoMet, the reductively pre-incubated enzyme displays a low activity of ≈ 5 m-units · mg^{-1} (Table 4, experiment 5). This basal activity is observed routinely and may be due to the fact that a small fraction of the purified enzyme contains bound AdoMet. The basal activity was stimulated by the addition of 5′-deoxyadenosine or of both 5′-deoxyadenosine and methionine (Table 4, experiments 7 and 8). Methionine alone is slightly inhibitory (Table 4, experiment 6).

Spectroscopic evidence of [Fe-S] cluster

The presence of equimolar amounts of iron and sulphide, as well as the long-wavelength absorbances in the UV-visible spectrum of the enzyme, indicates the presence of iron-sulphur cluster. To examine this issue, the X-band EPR spectra of various enzyme samples were obtained at 10 K. As shown in Figure 3(A), the purified enzyme displays an EPR signal at 10 K with an average g value of 2.01. The spectrum is similar to one observed in the clostridial enzyme [25] and assigned as a species of $[4Fe\text{-}4S]^{3+}$ [11]. The measured spin concentration ranges from 15 to 25 % of the enzyme concentration, differing from preparation to preparation. Addition of dithionite directly to the sample does not cause any noticeable change to the spectrum; however, reductive pre-incubation with cysteine converts the enzyme into an EPR-silent form (Figure 3B), presumably due to reduction of the cluster from $[4Fe\text{-}4S]^{3+}$ to $[4Fe\text{-}4S]^{2+}$.

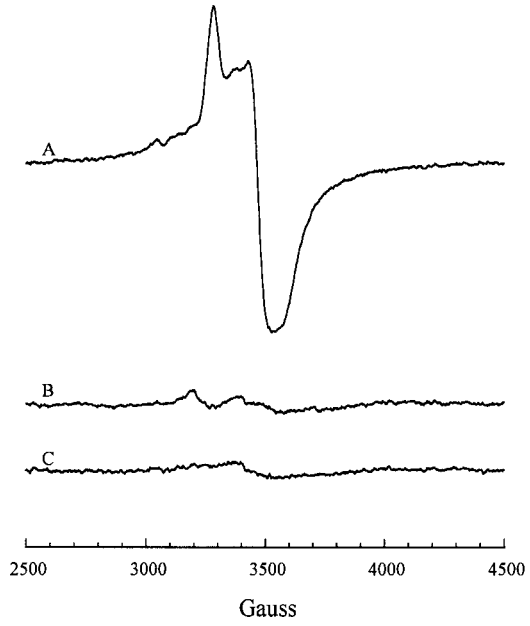


Figure 4 A comparison of AdoMet-dependent reduction of the iron-sulphur clusters in *Clostridium* and *B. subtilis* lysine 2,3-aminomutases

Shown are X-band EPR spectra acquired at 10 K. (**A**) 40 μM *Clostridium* enzyme plus 1.8 mM AdoMet and 4.0 mM dithionite; (**B**) 60 μM *B. subtilis* enzyme plus 1.8 mM AdoMet and 4.0 mM dithionite; (**C**) 60 μM *B. subtilis* enzyme plus 1.8 mM AdoHCys and 4 mM dithionite. Enzyme samples were subjected to reductive activation with dihydrolipoate for the *Clostridium* enzyme and with cysteine for the *B. subtilis* enzyme before being further treated with dithionite and AdoMet or dithionite and AdoHCys. Spectra were recorded at 9.244 GHz, with a modulation of 8.0 G and 1.0 mW power. Receiver gains were 5.0×10^3 for all samples. Spectra shown are averages of three 4-min scans with a time constant of 1.0 s.

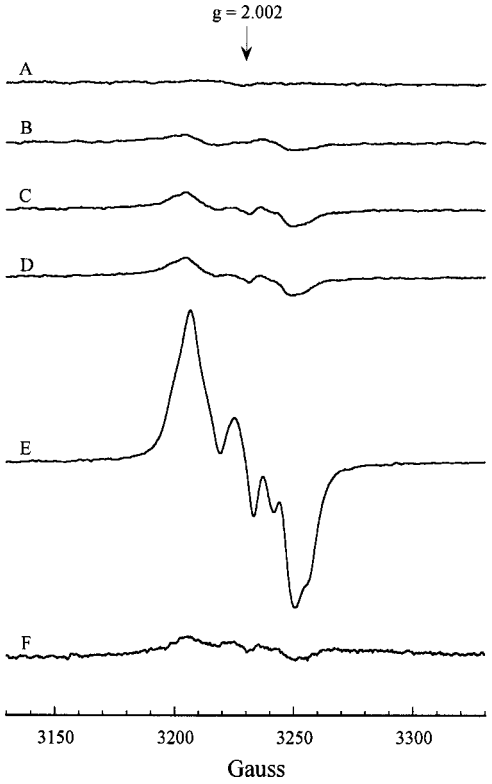


Figure 5 EPR spectra of organic radicals associated with *B. subtilis* lysine 2,3-aminomutase

All samples contained $\approx$ 60 μM enzyme (tetramer concentration) and in 100 mM Hepps buffer at pH 8.0 and 77 K. Spectra: (**A**) activated enzyme; (**B**) activated enzyme plus 67.2 mM lysine; (**C**) activated enzyme plus 67.2 mM lysine and 2.0 mM AdoMet; (**D**) activated enzyme plus 67.2 mM lysine and 2.5 mM dithionite; (**E**) activated enzyme plus 67.2 mM lysine, 2.0 mM AdoMet and 2.5 mM dithionite; (**F**) purified enzyme. Instrument settings: scan range, 200 G; centre field, 3300 G; microwave frequency, 9.093 GHz; modulation amplitude, 3.2 G; microwave power, 5.0 mW; receiver gain, 1.0×10^5.

In the case of the clostridial enzyme, the $[4Fe\text{-}4S]^{2+}$ cluster of the reductively pre-incubated enzyme can be further reduced to $[4Fe\text{-}4S]^{+}$ by dithionite in the presence of AdoMet or AdoHCys, with the concomitant appearance of a strong EPR signal of average $g = 1.85$ [11], as shown in Figure 4(A). In contrast, mixing the reductively pre-incubated *B. subtilis* enzyme with dithionite and AdoMet or AdoHCys elicits only weak, broad EPR signals (Figures 4B and 4C), which may represent a very low concentration of $[4Fe\text{-}4S]^{+}$. Like the clostridial enzyme, the reductively pre-incubated *B. subtilis* enzyme requires a strong one-electron reducing agent such as dithionite for activity, indicating that a further reduction of the enzyme is needed. Therefore, in both enzymes, the reduction of the cluster to the $[4Fe\text{-}4S]^{+}$ state seems to be a prerequisite for initiating the rearrangement reaction. The very low accumulation of the $[4Fe\text{-}4S]^{+}$ species in the *B. subtilis* enzyme may represent less stabilization of the reduced state by the protein environment relative to the 2+ state and may suggest a lower reduction potential for the $[4Fe\text{-}4S]^{2+}/[4Fe\text{-}4S]^{1+}$ couple in the *B. subtilis* relative to the clostridial enzyme.

EPR spectra of substrate-based free radicals

The presence of PLP together with the requirement for AdoMet suggests that the *B. subtilis* enzyme functions by a radical rearrangement mechanism similar to that proposed for the *Clostridium* enzyme (Scheme 1). To determine whether organic radicals could be observed, various enzyme–cofactor–substrate mixtures were examined by EPR at 77 K. Samples of the complete reaction mixture, including reductively pre-incubated *B. subtilis* enzyme, L-lysine, dithionite and AdoMet, reproducibly exhibit an EPR signal centred at $g = 2.002$ (Figure 5E). The g value of 2.002 and the partially resolved hyperfine structure indicate that the EPR signals are derived from an organic free radical. Indeed, the spectral envelope is very similar to that of the α-radical form of the β-lysine-PLP external aldimine (radical **3** in Scheme 1) observed in the steady state of the reaction of the clostridial lysine 2,3-aminomutase [6]. The spectral similarities suggest that the hyperfine coupling constants for the radical are similar in the *B. subtilis* and clostridial enzymes.

A series of control experiments show that the EPR signal is associated with enzyme activity and not with a by-product of the reductive pre-incubation. The spectrum does not appear in samples containing only the reductively pre-incubated enzyme (Figure 5A). Very weak signals were observed in samples lacking AdoMet and dithionite (Figure 5B), samples lacking dithionite alone (Figure 5C) and samples lacking AdoMet alone (Figure 5D). The presence of weak EPR signals in these control samples is attributed to the presence of a low concentration of lysine added as an enzyme stabilizer to buffers used in purification. Mixing the purified enzyme with lysine, dithionite and AdoMet does not produce the EPR signal either (Figure 5F). These observations are consistent with activity assays and indicate that the appearance of a strong EPR signal and a high activity of β-

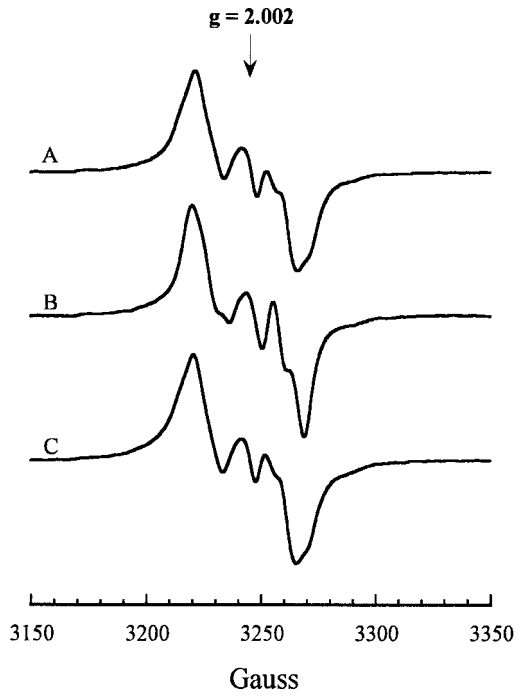


Figure 6 Effects of deuterium-labelled substrate on the EPR spectrum

All samples were prepared as described in Figure 5 except for the listed isotopic substitutions. Spectra: (**A**) reaction with L-lysine; (**B**) reaction with L-[3,3,4,4,5,5,6,6-$^2H_8$]lysine (99% enrichment); (**C**) reaction with L-lysine in 75% ^2H_2O. Instrument settings were identical to those listed in Figure 5 except the centre field was 3250 G.

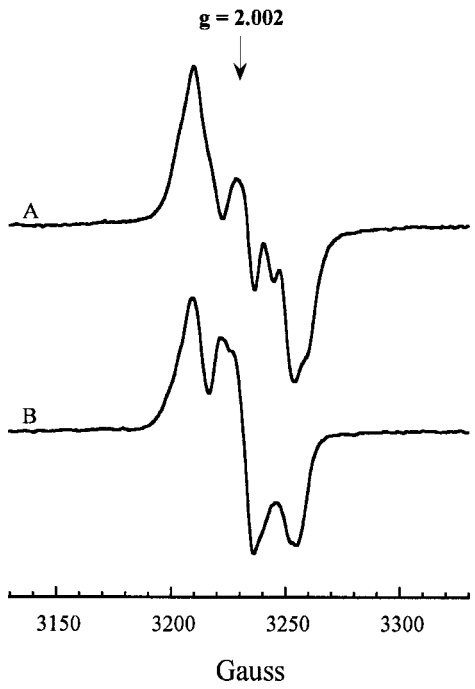


Figure 7 Effect of substrate analogue on the EPR spectrum

Activated *B. subtilis* lysine 2,3-aminomutase was mixed with either lysine or 4-thialysine in EPR tubes and then frozen in liquid nitrogen within 3 min. (**A**) The reaction mixture contained 50 mM activated enzyme, 67.2 mM lysine, 2.5 mM sodium dithionite and 2.0 mM AdoMet in 100 mM Hepps buffer at pH 8.0; (**B**) the reaction mixture contained 67.2 mM 4-thialysine in place of lysine in the same reaction mixture. The spectra were obtained at 77 K. Instrument settings: microwave frequency, 9.093 GHz; modulation amplitude, 1.6 G; microwave power, 5 mW; receiver gain, 1.0×10^5.

lysine formation require the fulfilment of the same conditions; that is, reductively pre-incubated enzyme and the presence of substrate, a strong one-electron reducing agent and AdoMet.

The EPR spectrum obtained from a reaction mixture with L-[3,3,4,4,5,5,6,6-$^2H_8$]lysine (Figure 6B) exhibits small but definite differences compared with that of unlabelled lysine (Figure 6A). These spectra show that one or more protons at C-3–C-6 of the lysine are coupled to the radical centre, most probably the C-3-proton as found for the corresponding radical in the clostridial enzyme [6]. The persistence of the predominant splitting pattern in the deuterated sample shows that protons from C-3 to C-6 of lysine are only weakly coupled to the unpaired electron spin and are not responsible for the major hyperfine splitting pattern in the spectrum. The effect of deuterium substitution in the solvent was also examined. The spectrum resulting from a reaction in 75% ^2H_2O is shown in Figure 6(C). The absence of any change in the splitting pattern indicates that solvent-exchangeable hydrogens are not strongly coupled to the radical.

4-Thialysine is an alternative substrate for clostridial lysine 2,3-aminomutase [26]. Upon mixing 4-thialysine with the reductively pre-incubated *B. subtilis* lysine 2,3-aminomutase in the presence of dithionite and AdoMet, an EPR signal centred at $g = 2.002$ was observed (Figure 7B). The signal is similar to that of the β-radical of 4-thialysine observed in the clostridial enzyme [26]. The similarities in the hyperfine structures of the spectra of organic free radicals derived from lysine and 4-thialysine bound to the *B. subtilis* lysine 2,3-aminomutase to those observed with the clostridial enzyme confirm that the reaction mechanisms are the same, and the conformations of the α- and β-substrate-based radicals are the same in the two enzymes.

Dependence of spin concentration on enzyme activity

The unpaired spin concentration at the $g = 2.0$ region depends on the concentration of the active enzyme, as shown in Figure 8. In preparing EPR samples, high concentrations of enzymes were used in both the pre-activation process and the filling of EPR sample tubes (see the Materials and methods section). In assays at high enzyme concentrations, the measured specific activities are severalfold lower than when the enzyme is assayed under standard conditions of low concentration (see Figure 8 and compare data in Table 3). An inverse correlation between enzyme concentration and specific activity at high enzyme concentrations was observed reproducibly (results not shown). However, the concentration of the unpaired electron spin was well correlated with the enzymic activities of the samples. The data indicate further that the observed radical is associated with enzyme that is actively turning over the substrate and that the radical is a genuine intermediate.

The product of the unknown *E. coli* gene P39280

In the absence of knowledge about the dioxygen-sensitivity of the unknown *E. coli* gene product, the protein was expressed in *E. coli* cells grown anaerobically. SDS/PAGE of cellular extracts demonstrated a very high level of expression of this gene in its natural host. No lysine 2,3-aminomutase activity could be detected, either in cellular extracts or in the purified protein, with

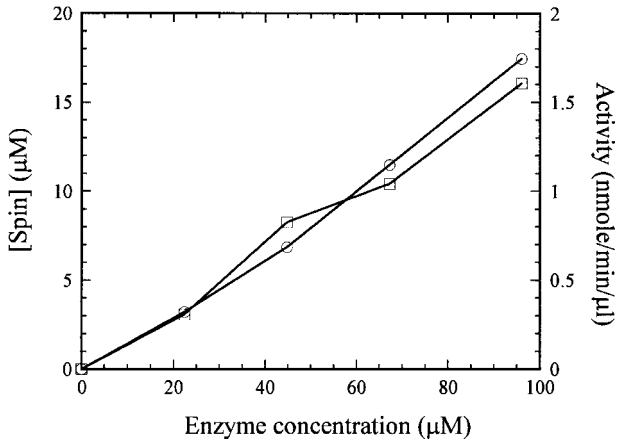


Figure 8 Correlation of spin with enzyme concentration and activity

All samples contained 100 mM Hepps buffer at pH 8.0, 67.2 mM lysine, 2.5 mM sodium dithionite and 2.0 mM AdoMet, in addition to components carried over from the pre-activation incubation along with the enzyme. Activity was measured by radiochemical assay of the same activated enzyme at concentrations identical to those used to generate EPR samples. □, Specific enzymic activity; ○, spin concentration.

or without reductive pre-incubation. This suggested a different and still unknown biological activity for this protein. The solid line in Figure 2 is the UV-visible absorption spectrum of the P39280 gene product. It includes a band at 420 nm and a long-wavelength tail, indicative of an iron sulphide cluster. Analysis for iron and sulphide confirmed their presence in equimolar amounts corresponding to about 3.0 mol per mol of subunits. This is in accord with the presence of the cysteine motif in the amino acid sequence (Figure 1A). The spectrum was similar to that of lysine 2,3-aminomutase but slightly less intense in the region of 400–430 nm, perhaps because of the absence of PLP. It was concluded that the *E. coli* protein of unknown function was not a lysine 2,3-aminomutase.

DISCUSSION

Oxygen insensitivity and low activity of *B. subtilis* lysine 2,3-aminomutase

Lysine 2,3-aminomutase from *C. subterminale* SB4 is extremely oxygen sensitive. Purification of the clostridial enzyme must be carried out under strictly anaerobic conditions to ensure maximal activity because exposure to dioxygen leads to irreversible destruction of the iron-sulphur cluster. In contrast, the *B. subtilis* lysine 2,3-aminomutase can be expressed and purified under aerobic conditions without loss of activity or iron-sulphur clusters. The purified enzyme must still be reductively pre-incubated with L-cysteine under anaerobic conditions to display its maximum activity, but the iron-sulphur cluster is not irreversibly oxidized by dioxygen. Dioxygen insensitivity is a potential advantage for future mechanistic studies of the *B. subtilis* enzyme and for its practical use in the production of L-β-lysine.

Although the activity of the *B. subtilis* enzyme is $\approx 1.7\,\%$ that of the clostridial enzyme, the spin concentration associated with the radical intermediate under steady-state conditions is 20–25 % of the tetrameric enzyme concentration, comparable with the observed spin intensity for the clostridial enzyme [6]. Furthermore, a very small deuterium kinetic isotope effect of $k_H/k_D \approx 1.2$ was observed with L-[3,3,4,4,5,5,6,6-$^2H_8$]lysine in the reaction of the *B. subtilis* enzyme, whereas an isotope effect of 3.0 was observed for the clostridial enzyme [10]. The high spin concentration generated during turnover and the low primary kinetic isotope effect in the reaction of the *B. subtilis* enzyme suggest that there is a slow step after the hydrogen-abstraction step, presumably the release of β-lysine.

The catalytically functional states of the iron-sulphur cluster

A strong, one-electron reducing agent such as dithionite is needed for activity in both the clostridial and the *B. subtilis* lysine 2,3-aminomutase-catalysed reactions. The strong reducing agent activates clostridial enzyme by reducing the iron-sulphur cluster from the EPR-silent $[4Fe\text{-}4S]^{2+}$ to the fully reduced $[4Fe\text{-}4S]^{+}$, which displays a low-temperature EPR spectrum centred at 1.85 *g*. The reduction requires the presence of AdoMet or AdoHCys, indicating that the binding of AdoMet to the enzyme makes the reduction potential of the iron-sulphur cluster less negative [11]. The **E**-$[4Fe\text{-}4S]^{+}$/AdoMet is postulated to be in equilibrium with a low concentration of a radical-initiating form that contains the 5′-deoxyadenosyl radical and may be designated as **E**-$[4Fe\text{-}4S]^{2+}$/Met/Ado$^{\bullet}$. The radical rearrangement is initiated by action of the 5′-deoxyadenosyl radical in abstracting a hydrogen atom from C-3 of lysine, which is bound as its external aldimine with PLP (Scheme 1).

Although the *B. subtilis* enzyme also requires a strong reducing agent for activity, dithionite is less effective in reducing the iron-sulphur cluster to $[4Fe\text{-}4S]^{+}$ than in the case of the clostridial enzyme. Only a weak broad EPR signal centred at $g = 1.9$ could be detected in the presence of AdoMet or AdoHCys, whereas a strong EPR signal is observed with the clostridial enzyme under the same conditions (Figure 4). However, the low *g* value (1.9) and the breadth of the *B. subtilis* signal are characteristic of $[4Fe\text{-}4S]^{+}$ clusters. The fact that a strong reducing agent such as dithionite is required for activity by both enzymes suggests that $[4Fe\text{-}4S]^{+}$ is also the active state in the *B. subtilis* enzyme. The low concentration of the reduced form in the *B. subtilis* enzyme, with dithionite as the reducing agent, may mean that the reduction potential for $[4Fe\text{-}4S]^{2+} + e^{-} \rightarrow [4Fe\text{-}4S]^{+}$ is more negative than in the case of the clostridial enzyme. The low concentration of $[4Fe\text{-}4S]^{+}$ in the *B. subtilis* lysine 2,3-aminomutase may contribute to its low activity relative to the clostridial enzyme.

On reductive pre-incubation

Although the purified *B. subtilis* lysine 2,3-aminomutase displays a trace of activity, reductive pre-incubation with L-cysteine increases it more than 15-fold. The increase is not directly correlated with the amount of the $[4Fe\text{-}4S]^{3+}$ state in the purified enzyme, indicating that L-cysteine does more than simply reduce the iron-sulphur cluster to $[4Fe\text{-}4S]^{2+}$. The requirement for L-cysteine in the pre-activation mixture suggests that cysteine may act as both a reducing agent and an activator in helping to position the iron-sulphur clusters in a reactive state. A possible function of L-cysteine is to remove inhibitory ligands adventitiously bound to the active site, perhaps to the iron-sulphur cluster, during cell growth and enzyme purification.

Effect of 5′-deoxyadenosine on activity

The data in the last four lines of Table 4 show that, in the absence of added AdoMet, 5′-deoxyadenosine significantly activates the enzyme. Under these conditions, 5′-deoxyadenosine is postulated to activate by increasing the amount of endogenous AdoMet. This could occur if part of the AdoMet sites were non-pro-

ductively occupied by methionine in a poised state from which 5′-deoxyadenosine had escaped. Reduction of the iron-sulphur cluster by dithionite in the presence of exogenous 5′-deoxyadenosine could lead to the formation of AdoMet, thereby activating those sites. The modest extent of activation by 5′-deoxyadenosine in Table 4 indicates that only a small percentage of the active sites could exist in such a poised state.

Significance of lysine 2,3-aminomutase associated with the *yod* operon

The *yodO* gene lies between *yodP* and *yozE* within a gene cluster of the *Bacillus* genome [15]. This cluster includes *yodT*, *yodS*, *yodR*, *argE*, *yodP*, *yodO* and *yozE* in an orientation in which *yodT* is transcribed first and *yozE* is last. The products of *yozE* and *yodP* are not similar to other known proteins. The *argE* gene encodes acetylornithine deacetylase for arginine biosynthesis, while the products of *yodR*, *yodS* and *yodT* are similar to butyrate:acetoacetate-CoA transferase, 3-oxoadipate-CoA transferase and adenosylmethionine:8-amino-7-oxononanoate aminotransferase, respectively. From this information, and our observation that the *yodO* protein does not accept either arginine or ornithine as aminomutase substrates, the relationship between the *Bacillus* lysine 2,3-aminomutase and other proteins encoded by this cluster cannot be ascertained at the present time.

The homologous product of *E. coli* gene P39280

The biological function of the *E. coli* gene product is unknown. Although it shares significant sequence identity with the clostridial and *B. subtilis* lysine 2,3-aminomutases (30%), residues that may be important for PLP binding, including a lysine residue, are absent, and PLP cannot be detected in the purified protein. Therefore, despite being homologous to lysine 2,3-aminomutase, the *E. coli* protein cannot be an aminomutase.

The gene is located in the *E. coli* genome adjacent to *efp*, encoding elongation factor P involved in the control of protein synthesis [27], and downstream from *groES* and *groEL* (*mopA*), genes which code for chaperonin proteins [28,29]. The fact that these genes do not code for enzymes directly or indirectly involved in lysine metabolism further supports a different biological role for this protein.

Other homologues of lysine 2,3-aminomutase

Among the seven homologues of *C. subterminale* lysine 2,3-aminomutase in Figure 1, two in addition to the *Bacillus yodO*-product are likely to be lysine 2,3-aminomutases. All of the homologues contain the cysteine motif (Figure 1A), but only the upper four contain the PLP-binding motif (Figure 1B). Those that include the PLP-binding motif are likely to be lysine 2,3-aminomutases. In particular, the homologous protein in *P. gingivalis* is 72% identical to *C. subterminale* lysine 2,3-aminomutase. The lower three homologues, including the *E. coli* protein, lack the PLP-binding motif and are unlikely to be lysine 2,3-aminomutases. The homologous protein in *A. aeolicus* includes a sequence that is similar to and aligned with the PLP motif in the other proteins, but with lysine and arginine in reversed positions. This protein cannot be assigned with confidence as a lysine 2,3-aminomutase, although the possibility should be regarded as open.

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