Activity and Spectroscopic Properties of the *Escherichia coli* Glutamate 1-Semialdehyde Aminotransferase and the Putative Active Site Mutant K265R†

Lawrence L. Ilag and Dieter Jahn*

Department of Molecular Biophysics and Biochemistry, Yale University, P.O. Box 6666, New Haven, Connecticut 06511

Received November 19, 1991; Revised Manuscript Received May 11, 1992

ABSTRACT: Glutamate 1-semialdehyde aminotransferase (glutamate 1-semialdehyde 2,1-aminomutase; EC 5.4.3.8; GSA-AT) catalyzes the transfer of the amino group on carbon 2 of glutamate 1-semialdehyde (GSA) to the neighboring carbon 1 to form δ-aminolevulinic acid (ALA). To gain insight into the mechanism of this enzyme, possible intermediates were tested with purified enzyme and the reaction sequence was followed spectroscopically. While 4,5-dioxovaleric acid (DOVA) was efficiently converted to ALA by the pyridoxamine 5′-phosphate (PMP) form of the enzyme, 4,5-diaminovaleric acid (DAVA) was a substrate for the pyridoxal 5′-phosphate (PLP) form of GSA-AT. Thus, both substances are reaction intermediates. The purified enzyme showed an absorption spectrum with a peak around 338 nm. Addition of PLP led to increased absorption at 338 nm and a new peak around 438 nm. Incubation of the purified enzyme with PMP resulted in an additional absorption peak at 350 nm. The reaction of the PLP and PMP form of the enzyme with GSA allowed the detection of a series of peaks which varied in their intensities in a time-dependent manner. The most drastic changes to the spectrum that were observed during the reaction sequence were at 495 and 540 nm. Some of the detected absorption bands during GSA-AT catalysis were previously described for several other aminotransferases, indicating the relationship of the mechanisms. The reaction of the PMP form of the enzyme with DOVA resulted in a similar spectrum as described above, while the spectrum for the conversion of DAVA by the PLP form of the enzyme indicated a different mechanism. To understand the functional significance of a conserved lysyl residue found in all cloned GSA-ATs, lysine 265 of *Escherichia coli* GSA-AT was changed to arginine by oligonucleotide-directed mutagenesis. The mutant enzyme K265R was overexpressed, purified to apparent homogeneity, and analyzed for its structural, catalytic, and spectroscopic properties. The enzymatic activity of K265R was only 2% of the wild-type enzyme activity, while its dimeric structure was not influenced by the mutation. The enzyme activity was stimulated by the addition of exogenous amines such as ethanolamine and methylamine. The spectrum of purified K265R showed significant absorbance only at 280 nm, indicating the absence of bound cofactor. The addition of PLP to K265R in the presence of ethanolamine prompted the formation of a peak at 438 nm, which was subsequently converted into a new peak at 338 nm. Further addition of GSA led to the conversion of the 338-nm peak into a peak at 305 nm. The nature of the catalysis performed by the mutant enzyme is different from the activity of the wild-type enzyme. To test the loss of GSA-AT function in vivo, the *hemL* gene, encoding GSA-AT, was disrupted by insertion of the Tc^r gene into its coding region. This *E. coli* strain has leaky ALA auxotrophy, indicating the presence of a compensatory pathway for ALA formation in *E. coli*. Transformation of the mutant strain with the wild-type gene restored normal growth, while transformation with the mutant gene encoding K265R resulted in drastically reduced growth but still differed from the control strain containing an empty plasmid.

In a variety of photosynthetic and nonphotosynthetic bacteria, including *Escherichia coli*, and in the chloroplasts of higher plants and algae, δ-aminolevulinic acid, a central precursor of porphyrin biosynthesis, is formed in a two-step reaction utilizing the five-carbon skeleton of glutamate [reviewed by Beale and Weinstein (1990); O'Neill & Söll, 1990; O'Neill et al., 1991; Jahn et al., 1992b]. Glutamate is activated by an aminoacyl bond to $tRNA^{Glu}$ (Jahn et al., 1992a). Glutamyl-tRNA is then the substrate of an NADPH-dependent reductase (glutamyl-tRNA reductase) which reduces the activated carboxyl group of glutamate to form GSA[1] or its cyclic form 2-hydroxy-3-aminotetrahydropyran-1-one (Chen et al., 1990; Jahn et al., 1991b; Houen & Gough, 1983; Jordan, 1990). Finally, GSA is the substrate for glutamate 1-semialdehyde aminotransferase (GSA-AT), which transaminates GSA to ALA. GSA-AT has been purified and characterized from barley, cyanobacteria, and green algae (Grimm et al., 1989; Jahn et al., 1991a; Rieble & Beale, 1991). The genes encoding the barley, *Synechococcus*, *Salmonella typhimurium*, *Bacillus subtilis*, and *E. coli* enzymes were cloned and characterized recently. The deduced amino acid sequences revealed a conserved peptide which is possibly involved in pyridoxal 5′-phosphate (PLP) binding (Grimm, 1990; Grimm et al., 1991a; Elliott et al., 1990; Hannson et al., 1991; Ilag et al., 1991, Figure 5). The cloned *E. coli* gene was overexpressed and its enzymatic function directly demonstrated (Ilag et al., 1991). Despite the structural similarities of the enzymes, there is still controversy about the exact mechanism of the GSA-AT. The involvement of PLP or PMP as a co-factor was questioned and several intermediates, such as 4,5-dioxovaleric acid (DOVA), 4,5-diamino-

† This work was supported by DOE and NIH grants to Dieter Söll.

* Author to whom correspondence should be addressed [telephone (203) 432-6204; fax (203) 432-6202].

[1] Abbreviations: ALA, δ-aminolevulinic acid; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; DAVA, 4,5-diaminovaleric acid; DOVA, 4,5-dioxovaleric acid; GSA, glutamate 1-semialdehyde; GSA-AT, glutamate 1-semialdehyde aminotransferase (glutamate 1-semialdehyde 2,1-aminomutase, EC 5.4.3.8); IPTG, isopropyl β-D-thiogalactopyranoside; PLP, pyridoxal 5′-phosphate, PMP, pyridoxamine 5′-phosphate; Tc^r, tetracycline resistance.

0006-2960/92/0431-7143$03.00/0

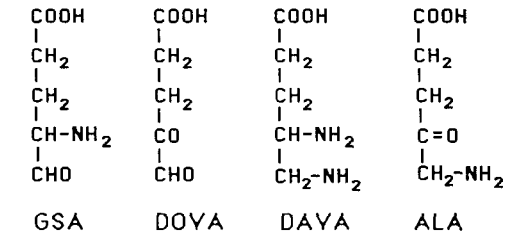

FIGURE 1: Possible intermediates during ALA formation from GSA. Shown are the structure of GSA and ALA, the substrate and product of the GSA-AT, and the structure of the two potential intermediates, DOVA (Breu & Dörnemann, 1988) and DAVA (Hoober et al., 1988).

valeric acid (DAVA), and the dimer of a GSA hydrate, were discussed (Grimm et al., 1989; Breu & Dörnemann, 1988; Hoober et al., 1988; Gough et al., 1989; see also Figure 1). In principle, a vitamin B_6 dependent transamination reaction could follow two different catalytic cycles. While the reaction of the PLP form of the enzyme with GSA would proceed through a DOVA intermediate, the reaction of the PMP form with GSA would generate DAVA as the corresponding intermediate.

Transaminases are a well-studied class of enzymes [reviewed in Christen and Metzler (1985); Hayashi et al., 1990]. The reaction pathway for several PLP enzymes has been studied using a wide variety of experimental approaches. On the basis of spectral, chemical, and kinetic investigations, it became evident that the half-reaction of the enzymatic transamination includes approximately eight sequential steps. These steps include the formation of an external aldimine, the removal of the α-proton to form quinonoid, the generation of the ketimine, and finally the hydrolysis of the latter, resulting in the release of the keto acid and the formation of the PMP form of the enzyme (Braunstein, 1985; Metzler et al., 1988). In aminotransferases, the cofactor PLP, usually attached to the enzyme via a Schiff base with a lysyl residue, facilitates the reaction by the electron-withdrawing nature of the pyridine moiety which labilizes the bonds around the C_α atom through the extended π-system of the Schiff base (Hayashi et al., 1990; Christen & Metzler, 1985). Nevertheless, the overall reaction efficiency and specificity are mainly determined by the functional groups of the protein. Therefore, a variety of transaminases were investigated for the contribution of single amino acid residues to the overall reaction using amino acid modifications or site-directed mutagenesis [reviewed in Hayashi et al. (1990)]. The mutation and modification of the active site lysyl residue responsible for the Schiff base formation to the cofactor resulted in a variety of different effects. While methylation of lysyl residue 258 of the aspartate-AT resulted in a total loss of activity, a spectral shift usually connected with the enzymatic reaction was still detected (Roberts et al., 1988). Substitution of alanine for the same residue completely abolished activity (Malcolm & Kirsch, 1985), while replacement of the lysyl residue with an arginine yielded an enzyme which still retained 2–3% activity compared to the wild-type enzyme (Kuramitsu et al., 1987). The activity of the mutated aspartate-AT (K258A) was improved by the addition of exogenous amines which functionally replaced the deleted catalytic group and acted as an acid/base catalyst for the 1,3 prototropic shift on the aldimine/ketimine interconversion (Toney & Kirsch, 1989). Similar experiments conducted with an active site mutant (K145Q) of the D-amino acid transaminase (Futaki et al., 1990) showed an increased rate of external aldimine formation upon addition of the exogenous amines, but the transformation to the pyridoxamine 5′-phosphate (PMP) form was unaffected.

To determine the basic mechanism of the GSA-AT, a series of enzymatic assays with different possible intermediates and GSA were conducted and analyzed via spectroscopy. The consequences of a site-directed mutation of a lysyl residue, believed to be involved in the Schiff base formation with the cofactor for the enzymatic activity in vitro and in vivo are described.

MATERIALS AND METHODS

Chemicals. All chemicals employed were of reagent grade from Sigma Chemical Co. unless stated otherwise. The potential reaction intermediate, 4,5-diaminovaleric acid (DAVA, DL-τ-ornithine), was also purchased from Sigma. 4,5-Dioxovaleric acid (DOVA) was a kind gift of Dr. D. Dörnemann, University of Marburg, Germany (Dörnemann & Senger, 1980). Glutamate 1-semialdehyde (GSA), synthesized by ozonolysis, was kindly provided by Dr. G. Kannangara, Carlsberg Research Laboratory, Copenhagen, Denmark (Gough et al., 1989). Sephadex G-50 and the FPLC resins Mono Q and Superose 12 were purchased from Pharmacia LKB Biotechnology.

Bacterial Strains and Plasmids. E. coli strain BL21(DE3), carrying the T7 RNA polymerase gene behind an IPTG inducible promoter (Studier et al., 1990), was used in protein overexpression (Ilag et al., 1991). The *hemL* gene was cloned into the plasmid pET3, which served as vector for overexpression of the *HemL* protein as described earlier (Ilag et al., 1991). Overexpression of the mutated *hemL* gene (K265R) in LI102 in the presence of ALA was performed at 30 °C analogous to the wild-type *hemL* gene. The starting strain for the mutation of the genomic *hemL* gene was *E. coli* JC7623 [*thr-1*, *ara-14*, *leuB6*, *delta(gpt-proA)62*, *lacY1*, *sbcC201*, *tsx-33*, *supE44*, *galK2*, *lambda⁻*, *rac⁻*, *sbcB15*, *hisG4*, *rfbD1*, *recB21*, *recC22*, *rpsL31*, *kdgK51*, *xyl-5*, *mtl-1*, *argE3*, *thi-1*; Kushner et al., 1971]. Since JC7623 cannot maintain plasmids, the mutation was transferred via P1 transduction into *E. coli* AB 354 [*thr-1*, *leuB6*, *panD2*, *lacZ4*, *glnV44(AS)*, *lambda⁻*, *rpsL8*, *thi-1*; Cronan, 1980] to create the new strain LI102. The mutant *hemL* gene was cloned into the plasmid pET3, which already served as vector for overexpression of the wild-type *HemL* protein in BL21(DE3) (Ilag et al., 1991). Overexpression of the mutant *hemL* gene in LI102 was achieved by cotransformation of pBP1-2, carrying the T7 RNA polymerase gene under the control of a thermoinducible lambda p_L promotor (Tabor & Richardson, 1985). The plasmid pPC1 is an Ap^r derivative of pBluescript KS^+, which carries the *hemL* gene on a 2.6 kb fragment cloned into the *Bam*HI and *Pst*I sites of the vector.

Construction of an E. coli Strain Carrying a Genomic Mutation in the GSA-AT Gene. Disruption of the *hemL* locus of *E. coli* by insertion of the Tc^r gene was performed after the methods of Jasin and Schimmel (1984) and Winans et al. (1985). A 1.4 kb *Eco*RI–*Ava*I fragment from pBR322 containing the Tc^r gene was blunt-ended by treatment with Klenow polymerase and cloned into the *hemL* gene of pPC1, which was digested previously in the middle with *Nhe*I. The resulting plasmid pPC4, in which the Tc^r gene is in the opposite orientation to the *hemL* gene, was linearized using *Xmn*I. *E. coli* JC7623 was subsequently transformed by electroporation with the linearized DNA. Since JC7623 has mutations in its *recB*, *recC*, and *sbcB* loci, it is transformable with linear DNA (Oishi & Cosloy, 1972; Wachernagel, 1973). Homologous double-recombination events were screened by testing for tetracycline resistance, ampicillin susceptibility, and ALA auxotrophy. Disruption of the genomic copy by the insertion

of the Tc^r gene was confirmed by Southern blot analysis using the *hemL* gene and the Tc^r gene as probes. Since JC7623 cannot maintain plasmids, the mutated *hemL* gene was transferred via P1 transduction to *E. coli* AB 354, resulting in strain LI102, carrying the mutation *hemL102* (Low, 1991). The strain LI102 has a leaky phenotype with regard to ALA auxotrophy, indicating the presence of a compensative pathway for the loss of *hemL* function.

Site-Directed Mutagenesis of GSA-AT. Mutagenesis of the *hemL* gene was performed in a pBluescript KS$^+$ derivative (pPC1) by the method of Kunkel (1985). To replace Lys265 with an Arg residue (K265R), the following oligonucleotide with the mutated bases underlined was chosen: 5′ CC-TGC-CTC-GGC-CGT-ATC-ATC-GGC-GG 3′. As the mutation created an *Eag*I site, restriction analysis with this enzyme was the basis for the initial screen for prospective mutants. The presence of the desired mutant was confirmed by DNA sequencing. To overexpress the mutant protein, an 800 bp *Hin*dIII–*Hpa*I fragment containing the K265R mutation was used to replace the homologous fragment of the wild-type *hemL* gene in pLIpopC, the plasmid used for the overexpression of the wild-type protein (Ilag et al., 1991).

Southern Blot Analysis. Chromosomal DNA was extracted from the *E. coli* strains LI102 and AB354 and digested with *Bam*HI and *Pst*I (Ausubel et al., 1987). The resulting DNA fragments were separated electrophoretically on a 0.8% agarose gel and transferred onto nitrocellulose membrane before the hybridization, washing, and autoradiography were performed as described earlier (Ausubel et al., 1987). The probe used first was the 2.6 kb *Bam*HI–*Pst*I fragment containing the *hemL* gene described above. The same blot was used for reprobing with another fragment containing the Tc^r gene liberated by an *Eco*RI–*Ava*I digest from pBR322. The previous probe was removed from the nitrocellulose filter as described. Radiolabeling of the probes was done by random priming as described (Ausubel et al., 1987).

Purification of the Recombinant GSA-ATs. Overexpression, extract preparation, and final purification by Mono Q chromatography were performed as outlined previously (Ilag et al., 1991). The mutant enzyme K265R was overexpressed in the deletion strain LI102 grown in the presence of ALA.

GSA-AT Assay. GSA-AT activity was measured with GSA as substrate unless stated otherwise as described earlier (Jahn et al., 1991a). Background values for the assay were reduced by purification of the substrate via Dowex 50-W chromatography prior use.

Spectroscopic Characterization. Absorption spectra were recorded at 22 °C on a Perkin-Elmer Lambda 2 spectrophotometer, with a recording speed of 480 nm/min in cells with 1-cm optical path. The purified enzyme was dialyzed overnight against 100 mM Bistris, pH 7.0, and was used in a final concentration of 40 μM. All reactions (1 mL) were carried out in 100 mM Bistris, pH 7.0, and additions were made in 2–50-μL volumes. For the analysis of the initial binding of both cofactors to the enzyme, 40 μM PLP or PMP was placed in the cuvette and their absorbance was set to zero before addition of the protein (in 50 μL). The substrate GSA and the intermediates DOVA and DAVA were added to final concentrations of 0.5, 2, and 0.4 mM, respectively. The exogenous amine ethanolamine was added to a final concentration of 10 mM. Absorbance was measured in cycles of 30 s unless stated otherwise.

RESULTS

Enzymatic Conversion of DOVA and DAVA to ALA by GSA-AT. Recent reports speculated about the possible role of DAVA and DOVA as intermediates during the transamination reaction from GSA to ALA (Breu & Dörnemann, 1988; Hoober et al., 1988; Bull et al., 1990). The availability of recombinant purified protein has allowed for the direct testing of the influence of these components on the GSA-AT reaction. Different concentrations of the potential intermediates DOVA and DAVA were tested for their ability to participate as substrates in the formation of ALA by GSA-AT. Since DAVA already contains two amino groups, one would expect its most efficient reaction to be with the PLP form of the enzyme. Therefore, it was not surprising that reactions with the PMP form of the enzyme did not yield a significant enzymatic conversion of DAVA to ALA. Table I shows that DAVA was an efficient substrate for the PLP form of GSA-AT. As expected, DOVA was not a substrate for the PLP form of the enzyme due to the lack of transferrable amino groups. In the presence of the PMP form of the enzyme, conversion of DOVA to ALA was detected. A reaction optimum was found when DOVA was present in approximately equimolar concentration to the enzyme. However, both substances are not as efficiently converted to ALA as are comparable amounts of GSA. Interestingly, high amounts of the enzyme, without prior incubation with cofactor, converted only DAVA to ALA, thus indicating that the PLP form of GSA-AT is present in vivo.

Table I: Enzymatic Conversion of DOVA and DAVA to ALA by GSA-AT[a]

added compounds	ALA formed (nmol)
GSA-AT (100 μM) + PLP + GSA (500 μM)	41.5
GSA-AT (100 μM) + PLP + DAVA (10 μM)	nd
GSA-AT (100 μM) + PLP + DAVA (50 μM)	nd
GSA-AT (100 μM) + PLP + DAVA (100 μM)	2.5
GSA-AT (100 μM) + PLP + DAVA (200 μM)	5.5
GSA-AT (100 μM) + PLP + DAVA (500 μM)	8.0
GSA-AT (100 μM) + PLP + DOVA (200 μM)	nd
GSA-AT (100 μM) + PMP + GSA (500 μM)	45.1
GSA-AT (100 μM) + PMP + DOVA (10 μM)	nd
GSA-AT (100 μM) + PMP + DOVA (50 μM)	3.1
GSA-AT (100 μM) + PMP + DOVA (100 μM)	5.5
GSA-AT (100 μM) + PMP + DOVA (200 μM)	4.2
GSA-AT (100 μM) + PMP + DOVA (500 μM)	2.0
GSA-AT (100 μM) + PMP + DAVA (200 μM)	nd
GSA-AT (500 μM) + – + DAVA (5 mM)	5.0
GSA-AT (500 μM) + – + DOVA (1 mM)	nd

[a] Assays (0.1 mL), containing GSA-AT (Mono Q fraction, indicated concentrations), 100 μM PLP or PMP (where indicated) and other additions shown above, were preincubated under standard conditions for 20 min at room temperature before GSA, DOVA, or DAVA in outlined concentrations was added, and the incubations were continued for another 20 min. Control reactions with heat-inactivated enzyme were performed for all reactions, and obtained background values (0.1–0.35 nmol of ALA) were subtracted from all determined values (nd, not detectable).

Spectral Properties of the GSA-AT. Due to the spectroscopic properties of the PLP/PMP cofactor, the mechanism of aminotransferases was intensively studied with a variety of spectroscopic methods [reviewed in Christen and Metzler (1985); Hayashi et al., 1990]. The enzymes absorb at a specific wavelength depending on the configuration of the bound cofactor (Metzler & Metzler, 1987). Moreover, for several PLP enzymes the absorbances of the various proposed intermediates of the reaction sequence have been assigned to defined regions of the spectrum (Braunstein, 1985; Metzler et al., 1988). To understand the relationship of the GSA-AT mechanism to the well-defined catalytic processes of other aminotransferases, a spectral analysis of the enzyme and its reaction sequenee was conducted. First, the purified recombinant GSA-AT was analyzed without further addition. As shown in Figure 2 a major peak at 280 nm, due to absorbance by the protein, and

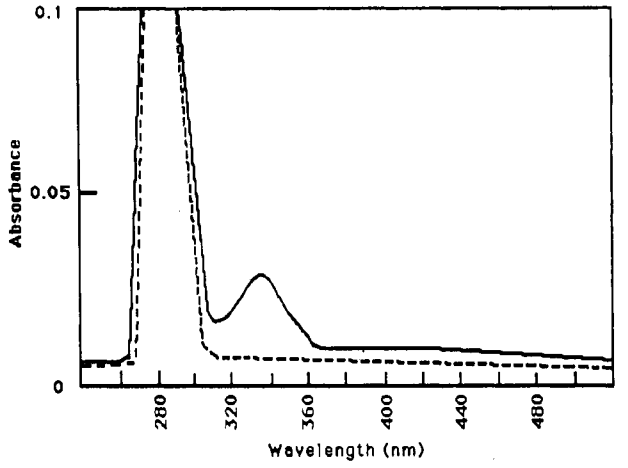

FIGURE 2: Absorption spectra of GSA-AT and the mutant enzyme K265R. The absorption of equal amounts of purified wild-type (—) and K265R (- - -) enzyme (40 μM Mono Q fraction) was recorded in 100 mM Bis-tris at 22 °C as outlined under Materials and Methods. The optical densities for the wild-type and the K265R enzyme at 280 nm peaked at a value of 0.22.

a second peak around 338 nm were detected. Since the functional analysis of untreated enzyme revealed the presence of PLP (Table I), one explanation for the absorption peak at 338 nm could be the formation of either the unprotonated internal aldimine or the enolimine tautomer of the protonated internal aldimine form of the enzyme, both absorbing approximately at this wavelength (Metzler et al., 1988; Higaki et al., 1991). The spectrum of the mutated enzyme (K273R) is described and discussed below.

Effects of PLP and PMP on the Spectrum of GSA-AT. Upon addition of PLP or PMP, a time-dependent change of the spectrum, previously recorded for the purified enzyme alone, was observed. The presence of PLP led to a small increase in the amplitude of the already present peak around 338 nm and generated, over a period of 2–3 min, a new peak around 438 nm. This latter peak falls within a region previously described as the region of the spectrum where the ketenamine tautomer of the protonated internal aldimine form of aminotransferases absorbs (Metzler et al., 1988; Higaki et al., 1991; Figure 3A). The PMP form of the enzyme was generated over a time period of 3–5 min after addition of PMP, as evidenced by a new absorption band at 350 nm (Figure 4A). The startup settings for the assays included the subtraction of the absorption bands for the free cofactors, so that their utilization due to binding to the protein produced a negative absorption at 395 (PLP) and 325 nm (PMP), respectively.

Kinetics of Spectral Changes of the PLP and PMP Form of GSA-AT upon Addition of GSA. The addition of GSA to both forms of the enzyme led to the time-dependent generation of several common peaks and a few unique absorption maxima. The absorption band for the PLP enzyme at 338 nm initially disappeared upon addition of GSA but then returned over a time period of 2 min to its original intensity, indicating the turnover of the active form of the PLP enzyme (Figure 3B). Moreover, the recovering absorption band in the 338-nm region was broadened to 330 nm, probably due to the parallel formation of the ketimine form of the enzyme. The peak around 438 nm changed only slightly over time with a slight shift to 430 nm. A common phenomenon for the complete transamination reaction, starting with the PLP enzyme, and for the half-reaction, starting from the PMP enzyme, was the presence of two dominant absorbance bands at 495 and 540 nm (Figures 3B and 4B). Both bands peaked after 1 min and

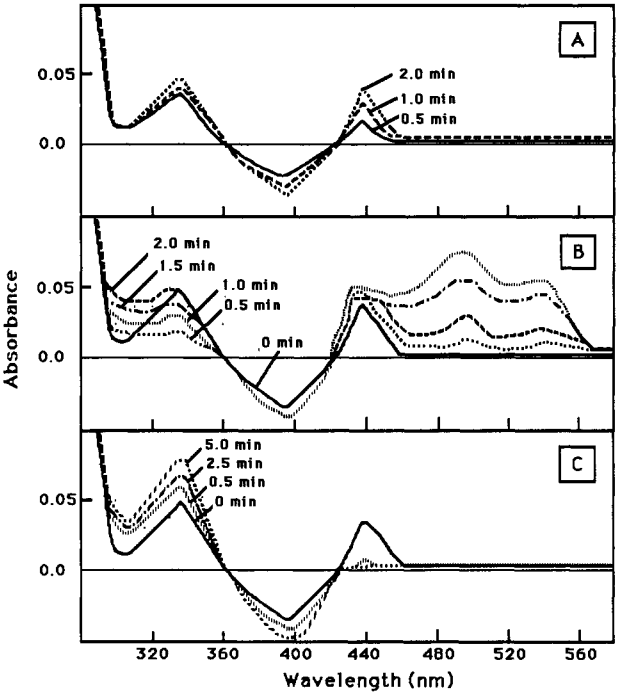

FIGURE 3: Effect of PLP binding and subsequent addition of GSA or DAVA on absorption spectrum of the purified recombinant GSA-AT from *E. coli*. Absorption spectra were taken every 30 s as outlined under Materials and Methods. Obtained absorption curves are labeled for the time point of record and plotted against the appropriate wavelength. (A) Incubation of purified GSA-AT (40 μM) at 22 °C with 40 μM PLP. The absorbance of the free PLP was subtracted before addition of the enzyme, and the difference spectrum is shown. (B) Addition of 0.5 mM GSA to the PLP enzyme shown in panel A. (C) Addition of 0.5 mM DAVA to the PLP enzyme shown in panel A.

subsequently decreased over a period of 4–10 min. Absorption in the 490–500-nm region of the spectrum was previously assigned to the quinonoid form of aminotransferases (Braunstein, 1985; Metzler & Metzler, 1987; Metzler et al., 1988). The accompanying band at 540 nm has not yet been described. Unique to the half-reaction with the PMP enzyme is the strong appearance of an absorbance band at 410–420 nm, a region of the spectrum to which absorbance of the external aldimine of several aminotransferases was assigned (Figure 4B; Metzler & Metzler, 1987; Higaki, 1991).

Effect of DAVA and DOVA on the Spectrum of GSA-AT. As described above, both DOVA and DAVA served as substrates for GSA-AT in the presence of the appropriate cofactor. Spectral analysis of their catalysis showed interesting differences. The reaction of the PLP enzyme with DAVA showed a time-dependent conversion of the absorbance band at 438 nm into a band at 338 nm without the detection of any intermediates (Figure 3C). This indicated that either the reaction sequence is very rapid, already completed before the first measurement at 30 s, and that the subsequent appearance of the 338-nm band represents a secondary reaction or that the reaction of the PLP form of the GSA-AT with DAVA follows a different type of enzymatic mechanism. However, the half-reaction of transamination performed with the PMP enzyme and the keto acid DOVA followed exactly the expected reaction sequence and was almost identical to the results obtained with GSA (Figure 4C). Upon addition of DOVA to the assay, the PMP form of the enzyme disappeared, giving rise to the transient formation of peaks at 495 and 540 nm and the generation of two final absorption bands in the 338- and 410-nm regions.

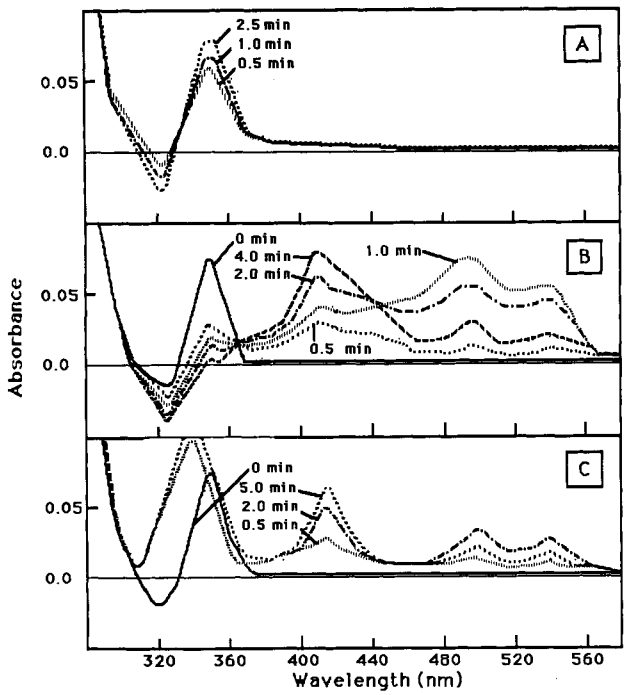

FIGURE 4: Effect of PMP binding and subsequent addition of GSA or DOVA on absorption spectrum of the purified recombinant GSA-AT from *E. coli*. Absorption spectra were taken every 30 s as outlined under Materials and Methods. Obtained absorption curves are labeled for the time point of record and plotted against the appropriate wavelength. (A) Incubation of purified GSA-AT (40 μM) at 22 °C with 40 μM PMP. The absorbance of the free PMP was subtracted before addition of the enzyme, and the difference spectrum is shown. (B) Addition of 0.5 mM GSA to the PMP enzyme shown in panel A. (C) Addition of 2.0 mM DOVA to the PMP enzyme shown in panel A.

Construction, Overexpression, Purification, and Structural Properties of the K265R Mutant of GSA-AT. It is well established that, in aminotransferases, the cofactor PLP is linked via a Schiff base with the amino group of a lysyl residue in the active site of the enzyme. Analysis of the amino acid sequences deduced from cloned genes encoding GSA-ATs and related aminotransferases revealed the presence of a highly conserved amino acid motif with a lysine as a central residue (see alignment in Figure 5). To investigate the significance for catalysis of this residue, lysine 265 of *E. coli* GSA-AT was changed to arginine by oligonucleotide-directed mutagenesis as detailed under Materials and Methods. The mutated enzyme (K265R) was overexpressed to the same extent as the wild-type enzyme using the IPTG-inducible T7 RNA polymerase system developed by Studier and co-workers (Studier et al., 1990; Ilag et al., 1991). K265R showed the same high affinity to the FPLC resin Mono Q as the wild-type GSA-AT and was therefore purified to apparent homogeneity in the same way using the earlier described one-step method (Ilag et al., 1991). While there was no obvious difference in the chromatographic behavior (almost identical elution point from Mono Q) of both proteins, the purified mutant GSA-AT seemed to be more sensitive to degradation, especially at temperatures above 33 °C, compared to the wild-type protein (data not shown). To investigate the consequences of the introduced mutation for the dimeric structure of the protein, gel filtration experiments were conducted. The purified K265R was only detected in its dimeric form. Moreover, the electrophoretic behavior of the mutant and that of the wild-type protein were almost identical when analyzed by two-dimensional gel electrophoresis (data not shown). These results indicated that the introduced mutation did not lead to drastic structural changes.

Spectroscopic Features of K265R. As shown in Figure 2, no significant absorption band from K265R was observed, except for the maximum at 280 nm, derived from the absorption of the protein itself. This is in contrast to the data obtained for the wild-type enzyme which showed the typical absorption pattern with a peak around 338 nm as expected for a PLP enzyme. We concluded from this result that the purified mutant enzyme did not contain detectable amounts of bound cofactor.

In Vitro Activity of K265R and the Influence of Exogenous Amines. A series of enzyme assays were conducted comparing the enzymatic activity of the wild-type enzyme with that of the mutant enzyme. The sensitivity of the assay was limited by the colorimetric nature of the test and the lack of an available radioactive substrate. Under the employed conditions, the wild-type enzyme showed a specific activity of approximately 5 μmol of ALA formed min^{-1} (mg of protein used).$^{-1}$. The use of high amounts of K265R (up to 1 mg) allowed a determination of an enzymatic activity of 100 nmol of ALA formed mg^{-1} min^{-1}, corresponding to 2% of the wild-type enzyme activity. Both activities were measured in the initial phase of velocity. As outlined in the introduction, addition of exogenous amine, in the form of ethanolamine or methylamine, can substitute for the mutated lysyl residue through its formation of a Schiff base with the enzyme bound cofactor. As shown in Table II, addition of both amines to K265R enhanced enzyme activity by a factor of 2, while the wild-type enzyme did not show any change in activity. Interestingly, preincubation of the mutant enzyme with PLP in the presence of exogenous amine for up to 1 h enabled the detection of activity even with lower amounts of protein, thus indicating the formation of an "active state" over several minutes (Figure 6). The wild-type enzyme did not show any changes in activity upon preincubation with PLP in the presence or absence of exogenous amines (Figure 6).

Effect of PLP, Ethanolamine, and GSA on the Absorption Spectrum of K265R. Incubation of K265R in the presence PLP resulted in the formation of an absorbance peak at 438 nm over a time period of 10 min. This peak was subsequently converted into a second peak at 338 nm after 1 h (Figure 7A). The positions of both observed absorbance bands were similar to those of bands detected for the reaction with the wild-type enzyme (Figures 3 and 4). But the clear differences in the time-dependent interconversion indicated a different type of mechanism. Previously found shifts of absorbance maxima due to differences in the absorbance between aldimines formed with the lysyl residue of the protein and with an exogenous amine were not detected (Futaki et al., 1990). Addition of PLP to the mutant enzyme in the absence of ethanolamine led to the slow generation of a small peak at 438 nm (20% of the peak generated in the presence of exogenous amines), which was not converted into anything else over a time period of 2 h (data not shown). Treatment of the wild-type enzyme under similar conditions with ethanolamine did not change the observed reaction sequence described above (data not shown). The area of negative absorbance approximately at 395 nm can be explained by the continuous utilization of PLP during the reaction. Further addition of GSA to mutant PLP enzyme led to the generation of a new 305-nm band, which was only discovered after the subtraction of the absorption spectrum for the PLP enzyme (Figure 7B). The significance of the slightly increased absorbance from 470 to 530 nm is not clear.

Human Ornithine AT	288 V L L G K A L S G 296	(Inana et al., 1986)
Rat Ornithine AT	288 V L L G K A L S G 296	(Muekler & Pitot, 1985)
E. coli 2-amino-3-ketobutyrate CoA ligase	240 G T L G K A L G G 248	(Mukherjee & Dekker, 1990)
Mouse ALA synthase	386 G T L G K A F G C 394	(Schoenhaut & Curtis, 1986)
R. capsulatus ALA synthase	254 G T L A K A Y G V 262	(Hornberger et al., 1990)
Barley GSA-AT	305 T T L G K I I G G 313	(Grimm, 1990)
Synechococcus GSA-AT	271 T T L G K I I G G 279	(Grimm et al., 1991a)
B. subtilis GSA-AT	264 T C L G K V I G G 272	(Hannson et al., 1991)
S. typhimurium GSA-AT	262 T C L G K I I G G 270	(Elliott et al., 1990)
E. coli GSA-AT	261 I C L G K I I G G 269	(Grimm et al.,1991a)

FIGURE 5: Alignment of conserved amino acid sequences around the potential PLP binding lysyl residue from different aminotransferases. The putative lysines are underlined. Lysine 265 of the *E. coli* GSA-AT was changed to an arginine by site-directed mutagenesis.

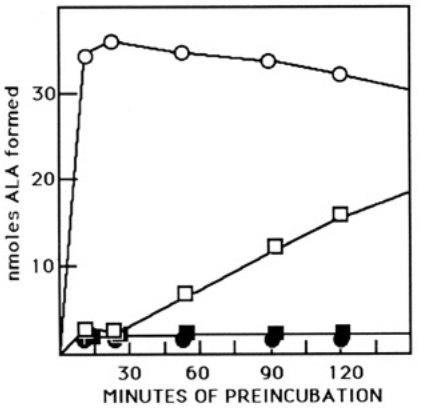

FIGURE 6: Preincubation of the mutant GSA-AT with PLP enhances enzymatic activity. Purified K265R (25 μM, □) and purified wild-type enzyme (5 μM, ○) were incubated at 22 °C in the presence of 100 μM PLP for the time points indicated. The subsequent enzymatic reaction for 15 min at 22 °C was initiated by the addition of 40 nmol of GSA. Reaction products were isolated and quantitated as described earlier (Jahn et al., 1991a). Reactions with boiled enzyme (■ for the mutant and ● for the wild-type) served as background controls.

Table II: Influence of Exogenous Amines on the Conversion of GSA to ALA by the Wild-Type and Mutant GSA-AT[a]

added compounds	ALA formed (nmol)
mutant GSA-AT (100 μM) +	
PLP (100 μM)	3.2
PLP (100 μM) + ethanolamine (10 mM)	5.6
PLP (100 μM) + methylamine (10 mM)	7.2
PMP (100 μM)	3.5
PMP (100 μM) + ethanolamine (10 mM)	6.5
PMP (100 μM) + methylamine (10 mM)	8.0
wild-type GSA-AT (5 μM) +	
PLP (100 μM)	34.6
PLP (100 μM) + ethanolamine (10 mM)	32.2
PLP (100 μM) + methylamine (10 mM)	33.2

[a] Assays (0.1 mL), containing purified mutant GSA-AT (Mono Q fraction, 100 μM protein) or purified wild-type GSA-AT (5 μM) and additions indicated above, were preincubated under standard conditions for 20 min at room temperature before 50 nmol of GSA was added, and the incubations were continued for another 20 min. Control reactions with heat-inactivated enzyme were performed for all reactions, and obtained background values (0.1–0.35 nmol of ALA) were subtracted from all determined values.

Analysis of the in Vivo Activity of K265R in Newly Constructed Strain Carrying an Inactivated Genomic Copy of the GSA-AT Gene. To test enzymatic activity of K265R in vivo, the genomic copy of GSA-AT was inactivated by the insertion of the Tc^r gene into the coding region as outlined under Materials and Methods. The mutation of the *E. coli* chromosome was checked by Southern blot analysis (Figure 8). The coding region of the GSA-AT was liberated from the chromosome of the strains LI102 (carrying the insertion) and AB354 (as control) by *Bam*HI and *Pst*I digest and probed with labeled DNA complementary to the coding region of the *hemL* gene. Since the inserted Tc^r gene already contained one *Bam*HI site, two fragments of approximately 2.3 and 1.7 kb were expected from the DNA of *E. coli* LI102, while the

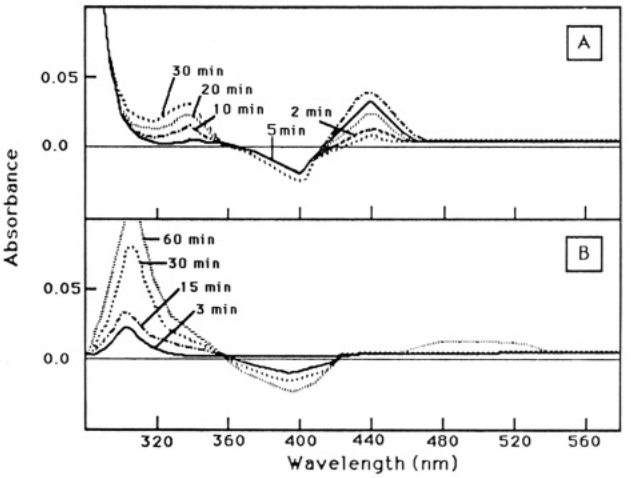

FIGURE 7: Effect of PLP binding and subsequent addition of GSA on the spectra of GSA-AT. Absorption spectra were taken at indicated time intervals as outlined under Materials and Methods. Obtained absorption curves are labeled for the time point of record and plotted against the appropriate wavelength. (A) Incubation of purified mutant GSA-AT (40 μM) at 22 °C with 40 μM PLP and 10 mM ethanolamine. The absorbance of the free PLP was subtracted before addition of the enzyme, and the difference spectrum is shown. (B) Addition of 0.5 mM GSA to the mutant PLP enzyme shown in panel A. The spectrum shown in panel A was subtracted prior addition of GSA, and the difference spectrum is shown.

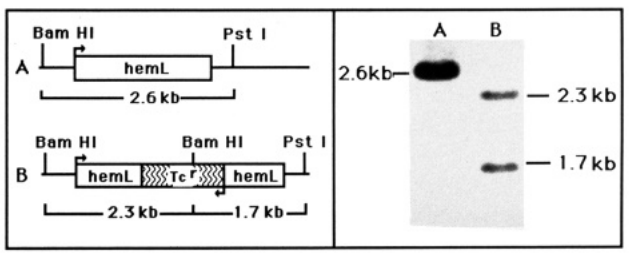

FIGURE 8: Southern blot analysis of the disrupted genomic copy of the GSA-AT gene. Isolated chromosomal DNA from the *E. coli* strains LI102 (lane 2) carrying the Tc^r gene inserted into the coding region of the GSA-AT gene and as control DNA from AB354 (lane 1) were digested with *Bam*HI and *Pst*I and probed with the 2.6 kb *Bam*HI/*Pst*I fragment containing the GSA-AT (*hemL*) gene. The autoradiogram of the experiment and a graphic explanation are shown.

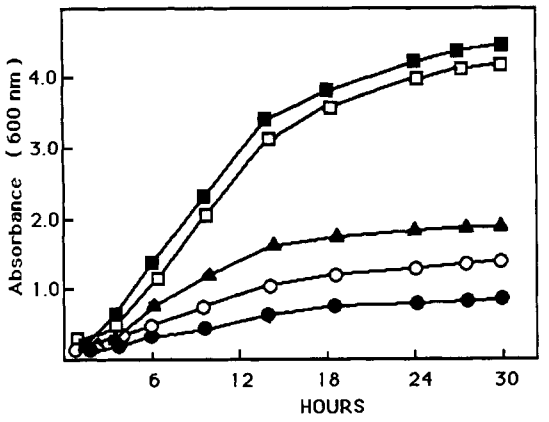

FIGURE 9: Growth curves of *E. coli* strains with intact (AB354) and disrupted genomic *hemL* gene copy (LI102) transformed with cloned wild-type *hemL* gene (pLIpopC), the K265R mutation carrying *hemL* gene (pLIK265R), and empty expression vector (pET3). The bacteria were grown at 30 °C in minimal media containing 12 g/L casamino acids, 0.3 mM Thr, 0.3 mM Leu, 0.1 mM pantothenic acid, 0.2% glycerol, and the appropriate antibiotics for selection in the following combinations: ■, = AB354 + pET3; □, = LI102 + pLIpopC; ○, = LI102 + pLIK265R; ▲, = LI102 + pLIK265R + pGP1-2 (carrying T7 RNA polymerase gene), grown at 33 °C; ●, = LI102 + pET3.

digested DNA from the control strain AB354 should lead to a single band of 2.6 kb. The Southern blot in Figure 8 confirmed the structure of the mutated genomic copy of the *hemL* gene. Probing of the same blot with a labeled probe complementary to the Tc^r gene resulted in the labeling of the two fragments of LI102 DNA, while no signal was detected with the DNA of the control strain AB354. This clearly demonstrated that a single copy of the Tc^r gene was inserted into the genome of LI102 (data not shown). The phenotype of the mutant was found to be leaky with respect to ALA auxotrophy, as indicated by the slow growth of the strain (colonies were detected only after 2–3 days). Further analysis by Southern blot and DNA sequence determination demonstrated that this phenotype was not the result of reversion but rather an alternative mechanism of ALA formation. Transformation of the strain with a plasmid carrying the GSA-AT gene rescued wild-type growth of the strain, while transformation with the mutant gene only resulted in slow growth, which was still faster than with the mutant strain carrying an empty plasmid (Figure 9). Using the T7 RNA polymerase promotor of the cloning vector by cotransformation of a heat-inducible T7 RNA polymerase gene, produced enough mutant enzyme in the cell to enhance growth (Figure 9). But faster growth of the overproducing strain was only detected at 33 °C, while at 37 °C the cell grew as slow as the mutant strain alone, thus indicating an instability of the mutant protein at higher temperature. The temperature-inducible lambda p_L promotor for T7 RNA polymerase induction has 20–30% of its maximum activity at 33 °C (Lowman & Bina, 1990). Moreover, a control strain without the T7 RNA polymerase-encoding plasmid did not show significant difference in growth when incubated at 30 and 33 °C, indicating that the observed enhanced growth of the T7 RNA polymerase gene-containing strain was due to overexpression of the mutant GSA-AT gene (data not shown). The results of the in vivo analysis led to the conclusion that reduction of the activity of the enzyme by the introduction of an active site mutation resulted in the loss of a major portion of its biological function in the cell. However, overproduction of the mutant enzyme led to the partial compensation of the catalytic defect and enabled the cell to grow at an almost normal rate.

DISCUSSION

The purified overexpressed wild-type GSA-AT catalyzed the conversion of DAVA but not DOVA into ALA, indicating the bound chromophore to be PLP. Similar results were recently obtained for *E. coli* aspartate-AT, which was found to be present mainly in its PLP form in the cell (Yagi et al., 1989). Nevertheless, in the presence of added appropriate cofactor (PLP for DAVA, PMP for DOVA) both intermediates were efficiently converted to ALA by GSA-AT. These findings raised the question for the role of PLP and PMP in the mechanism for GSA-AT. DOVA could be the first intermediate which appears after the formation of the PMP enzyme by the transfer of one amino group from GSA to the PLP enzyme. The enzymatic test demonstrated that addition of DOVA to the PMP enzyme led to the formation of ALA by utilizing a reaction sequence and an absorbance spectrum similar to the reaction with GSA. Moreover, Breu and Dörnemann (1988) showed, with titration experiment, that increasing amounts of DOVA inhibited the GSA-AT activity. The inhibition could be explained by blocking of the active site by the excess of DOVA. A reaction mechanism using DOVA would leave both possibilities for an intra- and intermolecular transfer of the amino group open. The reaction type would then be dependent on the release of the DOVA molecule after PMP enzyme formation. Mau and Wang (1988) proposed an intermolecular mechanism based on experiments with differentially labeled precursor molecules. DAVA reacted efficiently with the PLP enzyme to form ALA, thus creating a spectrum different from that of the reaction with GSA. Possible assignments for the different observed absorbance peaks are given throughout Results. The reaction of the PLP enzyme with DAVA might be either faster as the catalysis of GSA to ALA and the intermediates may be missed with the employed assay system or the mechanism is of a different nature. A different methodology might be needed to answer this question. Nevertheless, the fact remains that the enzyme was able to generate ALA from both substances, thus indicating that more work is needed to understand the in vivo situation.

Most known aminotransferases require the formation of a Schiff base between the cofactor and a lysyl residue of the enzyme for function [reviewed in Hayashi et al. (1990)]. Lysine 265 is part of a highly conserved amino acid sequence found in a variety of enzymes with similar function (Figure 5). Substitution of an arginine residue for the lysyl residue in position 265 of *E. coli* GSA-AT resulted in a change of the enzyme affinity to its cofactor PLP/PMP and in changed spectroscopic and catalytic properties. These results make the interaction of PLP with wild-type enzyme via the lysyl residue 265 very likely. No obvious conformational changes of the enzyme were detected, as was the case with asparate-AT carrying an analogous mutation (Kuramitsu et al., 1987). Nevertheless, the mutant enzyme was less stable at temperatures above 33 °C. Another similarity between the two mutant enzymes is their specific activity of approximately 2–3% of that of the wild-type enzyme. Kuramitsu et al. (1987) believe that the positive charge of the introduced arginine into the active site of the aspartate-AT (K258R) contributes to the enzymatic reaction or even changes its nature. The observed drastic changes in the spectra for the enzymatic reaction of the K258R mutant compared to that of the wild-type enzyme were similar to what we observed for our mutant. The overall activity of the mutant GSA-AT was enhanced by the presence of exogenous amines but did not lead to a significant improvement of the enzymatic activity as was

described for the mutant of aspartate-AT carrying an alanine residue at position 258 (K258A; Toney & Kirsch, 1989). No spectral shifts of the PLP form of the mutant GSA-AT in response to the addition of exogenous amines, as seen for mutant D-amino acid AT, were detected (Futaki et al., 1990). The difference in the effects of added amines to both mutated enzymes highlights a clear distinction between the two aminotransferases. The major absorbance peak at 305 nm which appeared after addition of GSA has not been observed for other aminotransferase systems. Interestingly, Metzler and Metzler (1987) described an absorption band in this region of the spectrum of aspartate-AT as a minor side peak which increased in the presence of amine buffer, but no clear assignment was given. Since a different enzymatic mechanism for the mutant GSA-AT is very likely, the observed spectra need further analysis before certain bands can be assigned to different forms of the enzyme. The detected low specific activity due to a different, less efficient, mechanism of the K265R mutant of GSA-AT showed clear consequences for cellular metabolism, almost abolishing efficient growth. As shown in vitro, high amounts of the mutant enzyme are able to compensate for catalytic inefficiency. This was confirmed in vivo by the enhanced growth of the mutant strain after overexpression of K265R. Interestingly, mutation of the genomic copy of the GSA-AT gene still allowed slow growth of bacteria. This implies that this compensatory alternative pathway can be connected with the presence of two forms of glutamyl-tRNA reductase (the first enzyme of the C5 pathway in *E. coli*) and with the efficient growth of a strain (*hemA*$^-$) carrying a mutation in one of the reductase genes under anaerobic conditions (Jahn et al., 1991b; Verkamp et al., 1992). An alternative explanation for the detected phenomenon is the conversion of GSA to ALA by another cellular aminotransferase with less substrate specificity. More structural data and further mutational studies are needed to understand the exact mechanism of the GSA-AT.

ADDED IN PROOF

During the review process of the manuscript, spectral and kinetic analyses of GSA-ATs from the photosynthetic organisms *Synechococcus* and pea have been published (Smith et al., 1991a,b; Pugh et al., 1992). Several of the obtained results are similar to the data presented above.

ACKNOWLEDGMENT

We thank Dr. Dieter Söll, in whose laboratory this work was carried out, for support and many discussions. We thank Drs. Martina Jahn and M. John Rogers for their help and interest. We are indebted to Dr. Carl Simmons, Dr. Lee Sylvers, and Albert Crescenzo for critical reading of the manuscript. We are indebted to Dr. Dieter Dörnemann, University of Marburg, Germany, and Dr. Gamini Kannangara, Copenhagen, Denmark, for the kind gifts of DOVA and GSA, respectively.

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1987) *Current protocols in molecular biology*, Greene Publishing Associates/Wiley-Interscience, New York.

Beale, S. I., & Weinstein, J. D. (1990) in *Biosynthesis of Heme and Chlorophylls* (Dailey, P. A., Ed.) p 287, McGraw-Hill, New York.

Braunstein, A. E. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) p 1, Wiley and Sons, New York.

Breu, V., & Dörnemann, D. (1988) *Biochim. Biophys. Acta 967*, 135.

Bull, A. D., Breu, V., Kannangara, C. G., Rogers, L. J., & Smith, A. J. (1990) *Arch. Microbiol. 154*, 56.

Chen, M.-W., Jahn, D., O'Neill, G. P., & Söll, D. (1990) *J. Biol. Chem. 265*, 4058.

Christen, P., & Metzler, D. E., Eds. (1985) *Transaminases*, Wiley and Sons, New York.

Cronan, M. (1980) *J. Bacteriol. 141*, 1291.

Dörnemann, D., & Senger, H. (1980) *Biochim. Biophys. Acta 628*, 35.

Elliott, T., Avissar, Y. J., Rhie, G.-E., & Beale, S. I. (1990) *J. Bacteriol. 172*, 7071.

Futaki, S., Ueno, H., Martinez del Pozo, A., Pospischil, M. A., Manning, J. M., Ringe, D., Stoddaed, B., Tanizawa, K., Yoshimura, T., & Soda, K. (1990) *J. Biol. Chem. 265*, 22306.

Gough, S. P., Kannangara, C. G., & Bock, K. (1989) *Carlsberg Res. Commun. 54*, 99.

Grimm, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4169.

Grimm, B., Bull, A., Welinder, K. G., Gough, S. P., & Kannangara, C. G. (1989) *Carlsberg Res. Commun. 54*, 67.

Grimm, B., Bull, A., & Breu, V. (1991) *Mol. Gen. Genet. 225*, 1.

Hannson, M., Rutberg, L., Schröder, I., & Hederstedt, L. (1991) *J. Bacteriol. 173*, 9740.

Hayashi, H., Wada, H., Yoshimura, T., Esaki, N., & Soda, K. (1990) *Annu. Rev. Biochem. 59*, 87.

Higaki, T., Tanase, S., Nagashima, F., Morino, Y., Scott, A. I., Williams, H. J., & Stolowich, N. J. (1991) *Biochemistry 30*, 2519.

Hoober, J. K., Kahn, A., Ash, D. E., Gough, S., & Kannangara, C. G. (1988) *Carlsberg Res. Commun. 53*, 11.

Hornberger, U., Liebetanz, R., Tichy, H.-V., & Drews, G. (1990) *Mol. Gen. Genet. 221*, 371.

Houen, G., & Gough, S. P. (1983) *Carlsberg Res. Commun. 48*, 567.

Ilag, L. L., Jahn, D., Eggertsson, G., & Söll, D. (1991) *J. Bacteriol. 173*, 3408.

Inana, G., Totsuka, S., Redmond, M., Dougherty, T., Nagle, J., Shiono, T., Ohura, T., Kominami, E., & Katunuma, N. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1203.

Jahn, D., Chen, M.-W., & Söll, D. (1991a) *J. Biol. Chem. 266*, 161.

Jahn, D., Michelsen, U., & Söll, D. (1991b) *J. Biol. Chem. 266*, 2542.

Jahn, D., O'Neill, G. P., Verkamp, E. & Söll, D. (1992a) *Plant Physiol. Biochem. 30*, 245.

Jahn, D., Verkamp, E., & Söll, D. (1992b) *Trends Biol. Sci. 17*, 215.

Jasin, M., & Schimmel, P. (1984) *J. Bacteriol. 159*, 783.

Jordan, P. M. (1990) in *Biosynthesis of Heme and Chlorophylls* (Dailey, H. A., Ed.) p 55, McGraw-Hill, New York.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.

Kuramitsu, S., Inoue, Y., Tanase, S., Morino, Y., & Kagamiyama, H. (1987) *Biochem. Biophys. Res. Commun. 146*, 416.

Kushner, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 824.

Low, K. B. (1991) *Methods Enzymol. 204*, 43.

Lowman, H. B., & Bina, M. (1990) *Gene 76*, 133.

Malcolm, B. A., & Kirsch, J. F. (1985) *Biochem. Biophys. Res. Commun. 132*, 915.

Mau, Y. H., & Wang, W.-Y. (1988) *Plant Physiol. 86*, 793.

Metzler, C. M., & Metzler, D. E. (1987) *Anal. Biochem. 166*, 313.

Metzler, C. M., Mitra, J., Metzler, D. E., Makinen, M. W., Hyde, C. C., Rogers, P. H., & Arnone, A. (1988) *J. Mol. Biol. 203*, 197.

Muekler, M. M., & Pitot, H. C. (1985) *J. Biol. Chem. 260*, 12993.

Mukherjee, J. J., & Dekker, E. E. (1990) *Biochim. Biophys. Acta 1037*, 24.

Oishi, M., & Cosloy, S. D. (1972) *Biochem. Biophys. Res. Commun. 49*, 1568.

O'Neill, G. P., & Söll, D. (1990) *Biofactors 2*, 227.
O'Neill, G. P., Jahn, D., & Söll, D. (1991) *Subcell. Biochem. 17*, 235.
Pugh, C. E., Harwood, J. L., & Robert, A. J. (1992) *J. Biol. Chem. 267*, 1584.
Rieble, S., & Beale, S. I. (1991) *Arch. Biochem. Biophys. 289*, 289.
Roberts, W. J., Hubert, E., Iriarte, A., & Martinez-Carrion, M. (1988) *J. Biol. Chem. 263*, 7196.
Schoenhaut, D. S., & Curtis, P. J. (1986) *Gene 48*, 55.
Smith, M. A., Grimm, B., Kannangara, C. G., & v. Wettstein, D. (1991a) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9775.
Smith, M. A., Kannangara, C. G., Grimm, B., & v. Wettstein, D. (1991b) *Eur. J. Biochem. 202*, 749.
Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074.
Toney, M., & Kirsch, J. P. (1989) *Science 243*, 1485.
Verkamp, E., Jahn, M., Jahn, D., Kumar, M., & Söll, D. (1992) *J. Biol. Chem. 267*, 8275.
Wachernagel, W. (1973) *Biochem. Biophys. Res. Commun. 51*, 306.
Winans, S. C., Elledge, S. J., Krueger, J. H., & Walker, G. C. (1985) *J. Bacteriol. 161*, 1219.
Yagi, T., Niu, S., Okawa, K., Yamamoto, S., & Nozaki, M. (1989) *Biochimie 71*, 427.