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Mutations at the Interdomain Hinge Region of the DadB Alanine Racemase: Effects of Length and Conformational Constraint of the Linker Sequence on Catalytic Efficiency†

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ABSTRACT: The pentapeptide "hinge" region of the DadB alanine racemase links two structural domains of the protein [Galakatos, N. G., & Walsh, C. T. (1987) *Biochemistry 26*, 8475]. The presence of substrate markedly reduces the rate of hinge-specific proteolysis of this racemase and induces a conformational change observed by circular dichroism. To evaluate the possible contribution of the proteolytically sensitive hinge residues (-Y^{253}GGGY^{257}-) on catalytic efficiency, site-directed mutations were generated to probe the effects of size and conformational rigidity of that region. A bacterial overproducing system for the *dadB* gene was constructed that expresses the enzyme as 4.5% of total soluble protein. On this construct, a four-part ligation allowed the engineering of two unique and proximal restriction sites required for cassette mutagenesis at the hinge region. For two of the eight mutants generated, expressed protein could not be detected (deletion of -GGGY-; termination codon at position 255). Deletion of one or two of the three Gly residues had no effect on catalytic efficiency. Insertion of a fourth Gly resulted in a 5-fold drop in V_{max}/K_m. For G254P, G255P, and G256P, V_{max}/K_m was 60%, 126%, and 26% of the native enzyme, respectively. In all cases, the K_m's remained essentially constant, suggesting that the hinge region is not involved in substrate binding. The rate of hinge-specific proteolysis of the mutants was faster than that of wild-type DadB except for the G255P protein for which it was equivalent.

It has been demonstrated that on average 26% of protein primary sequences are situated in loop regions (Leszcynski & Rose, 1986). For loops found in immunoglobulins and DNA-binding proteins, clear functional significance in recognition and binding has been assigned (Thornton et al., 1988). For enzymes, however, the role of particular loops in the control of catalytic efficiency or specificity is much less known. Surface, flexible portions of the polypeptide chain have recently been implicated in substrate or cofactor binding and as pivot areas for closure of interdomain active-site clefts (Huber, 1988). However, little direct experimental evidence has existed to support these hypotheses.

In some cases, site-directed mutagenesis has met with great success in elucidating aspects of the formidable catalytic efficiency of enzyme species (Knowles, 1987). Most of that work to date has targeted residues at the densely packed active-site region, which appears deceptively rigid. However, many enzyme proteins undergo ligand-induced conformational isomerizations, and thus effects of single-residue alterations can be masked by overall protein dynamics.

Bacterial alanine racemases catalyze the first committed step of peptidoglycan assembly in cell wall biosynthesis and have been of great interest mechanistically and therapeutically (Walsh et al., 1985). Previous work from these laboratories described the cloning, sequence determination, and purification from *Salmonella typhimurium* of two isoenzymic alanine racemases (Wasserman et al., 1984; Galakatos et al., 1986), one with a low k_{cat} (*alr* gene encoded) and the other (*dadB* gene encoded) with a 20–60-fold higher k_{cat} (Esaki & Walsh, 1986). Also reported is the analysis of the reaction profile energetics and the mechanism of inactivation of these PLP[1]-dependent racemases by natural and synthetic antibacterial agents (Faraci & Walsh, 1988).

The *S. typhimurium* racemases have so far proven refractory to crystallization, so in initial structure–function studies we have reported on the limited proteolysis of the DadB alanine racemase (Galakatos & Walsh, 1987). DadB was shown to possess a protease-labile loop region that connects two structural domains and is conserved in all four alanine racemase sequences reported from both Gram-negative and Gram-positive organisms. Proteolytic removal of the intraloop tetrapeptide -G^{254}GGY^{257}- from the 356-residue monomeric protein generates an enzyme species in which the two fragments remain associated. This "clipped" protein retains its

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[1] Abbreviations: CIP, calf intestinal alkaline phosphatase; dNTP, deoxynucleotide triphosphate; IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; DMT, 4′,4′-dimethoxytrityl; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; kb, kilobase; bp, base pair(s); Δ, residue deletion.

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secondary structure and 76% of intact active sites, but possesses only 3% of native catalytic activity.

In this paper the substrate-induced conformational isomerization of the DadB alanine racemase is studied and the function of the tetrapeptide "hinge" region of DadB is probed by site-directed mutagenesis. The effects of hinge length and conformational constraint on catalytic efficiency are evaluated, and analogies are drawn with other two-domain proteins known to require rigid-body movement for catalysis.

Materials and Methods

Materials. Escherichia coli strain W3110 lac I^Q was a kind gift of Dr. U. RajBhandary (MIT). *E. coli* JM101, M13mp18 RF DNA, deoxy- and dideoxynucleoside triphosphates, and deoxyadenosine 5′-(α-[^{35}S]thiotriphosphate) (>600 mCi/mmol) were purchased from Amersham. Expression vector pKK223-3 was obtained from Pharmacia. All restriction enzymes were from New England Biolabs. T4 DNA ligase was from BRL. Calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I, and X-Gal were obtained from Boehringer-Mannheim Biochemicals.

DMT phosphoramidites, solid-phase resins, and reagents for oligonucleotide synthesis were from Applied Biosystems Inc. IPTG was purchased from Bachem Inc. L-Alanine, NADH, PLP, pyridoxine monohydrochloride, phenylmethanesulfonyl fluoride (PMSF), and CHES were purchased from Sigma. Hog muscle lactate dehydrogenase (EC 1.1.1.27) (550 units/mg), D-amino-acid oxidase (EC 1.4.3.3) from hog kidney (15 units/mg), α-chymotrypsin, and subtilisin were obtained from Boehringer-Mannheim Biochemicals. Ultrapure SDS and Coomassie brilliant blue R-250 were from BRL. Acrylamide (>99.9%) was from Bio-Rad. All other chemicals were of analytical reagent grade.

Construction of Plasmid pNG21. The expression plasmid pNG21 is a subclone of the *dadB* gene in vector pKK223-3 (Pharmacia) that contains the tac promoter. Initially, the 1,4-kb *Mst*II–*Eco*RI fragment of pSW31 (Wasserman et al., 1984) was subcloned into pLC2833 (Galakatos et al., 1986). The resulting plasmid pED51 contains the *dadB* gene within the unique *Eco*RI–*Cla*I sites. pED51 was digested with *Cla*I, the ends were blunted, and the gene-containing fragment was isolated by treatment with *Eco*RI. This was then directionally ligated into the pKK223-3 vector that had been treated with *Eco*RI and *Sma*I and then phosphatased with CIP. This expression plasmid (pNG20) contained the tac promoter 140 bp upstream of the start codon of *dadB*. To generate pNG21, this distance was reduced to 39 bp by removal of the 5′-terminal *Eco*RI–*Mae*II fragment.

Oligonucleotide Synthesis. A total of 17 deoxyoligonucleotides (Table I) were synthesized at 0.2 μM scale by the phosphoramidite method on an Applied Biosystems Model 381A instrument. In all cases, the fully blocked, resin-bound oligomer was subjected to an additional DMT deblocking cycle and then was treated with 28% NH_4OH, first at 25 °C (1 h; removal from solid support) and then at 55 °C (12 h; side-chain deblocking). Preparative polyacrylamide denaturing gel electrophoresis followed by elution of the desired band with 0.1 M triethylammonium bicarbonate (TEAB), pH 7.2 (37 °C, 12 h), yielded homogeneous material. Finally, this was passed through a Millipore C18 Sep-Pak column, and the oligonucleotides were eluted with 50% CH_3CN/35 mM TEAB.

Construction of Oligonucleotide Cassettes. Equimolar amounts of pairwise-complementary oligonucleotides (Table I) that were purified as described above were incubated at 95 °C for 5 min in 100 mM Tris, pH 8.5, containing 100 mM $MgCl_2$. The reaction was performed at total oligonucleotide concentration of 1 μg/μL, and the mixture was then allowed to cool slowly to room temperature. The resulting double-stranded cassettes were diluted 10-fold with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and used directly in the ligation reactions.

Table I: Oligonucleotide Cassettes

oligonucleotide sequence	protein sequence	plasmid construct
5′-GGTACCCGTAGTTAAC-3′ 5′-TGAGTTAACTACGGGTACC-C-3′	-G^{252}YP*-	pNG41
5′-TCTGT-3′ 5′-ACAGAGTAC-3′	-Y^{253}S-	pNG421
5′-GGAGGGTATTCTGT-3′ 5′-ACAGAATACCCTCCGTAC-3′	-Y^{253}GGYS-	pNG422
5′-GGAGGGTATTCTGT-3′ 5′-ACAGAATACCCTCCGTAC-3′	-Y^{253}GYS-	pNG423
5′-GGAGGCGGAGGGTATTCTG-T-3′ 5′-ACAGAA-TACCCTCCGCCTCCGTAC-3′	-Y^{253}GGGGYS-	pNG424
5′-GGCCCGGGGTATTCTGT-3′ 5′-ACAGAATACCCCGGGCCG-TAC-3′	-Y^{253}GGPYS-	pNG440
5′-GGCGGACCGTATTCTGT-3′ 5′-ACAGAATACGGTCCGCCG-TAC-3′	-Y^{253}GPGYS-	pNG450
5′-CCGGGAGGGTATTCTGT-3′ 5′-ACAGAATACCCTCCCGGG-TAC-3′	-Y^{253}PGGYS-	pNG460
5′-ATAGCCATCGGCATAACCC-3′	sequencing primer	

Construction of Plasmid pNG41 (Template for Cassette Mutagenesis). Plasmid pNG41 is the template for cassette mutagenesis and possesses unique *KpnI* and *HpaI* sites positioned at the respective 5′- and 3′-termini of the encoded hinge region. These restriction sites were engineered by the following three-step process: Plasmid pNG21 was digested with *Eco*RI and *Hin*dIII, and the *dadB*-containing 1.4-kb fragment was isolated. This was then partially digested with *Hph*I, and the 0.9-kb *Eco*RI–*Hph*I fragment encoding the N-terminal domain of DadB was isolated. Similarly, treatment of the 1.4-kb *Eco*RI–*Hin*dIII fragment with *Dde*I allowed the isolation of the 0.5-kb *Dde*I–*Hin*dIII segment which corresponds to the C-terminal domain of DadB. Plasmid pNG41 was then constructed by a four-fragment ligation between the 0.9-kb *Eco*RI–*Hph*I and 0.5-kb *Dde*I–*Hin*dIII isolated fragments, the pNG41 linker (Table I), and the original vector for pNG21 that possesses 5′-*Eco*RI and 3′-*Hin*dIII ends (Figure 1). The construct was confirmed first by restriction analysis using the newly incorporated *Kpn*I site. Promising transformants were then treated with *Eco*RI and *Hin*dIII, subcloned into M13mp18, and sequenced by the Sanger method.

Construction of Site-Directed Cassette Mutants. Plasmid pNG41 was digested with *Kpn*I and *Hpa*I, and the 6.0-kb fragment was isolated. To this were added in separate reactions the respective mutagenesis cassettes and T4 DNA ligase, and the mixture was transformed into *E. coli* strain W3110. From each ligation reaction eight transformants were selected. These were screened directly for protein expression by growing 1.5-mL cultures, inducing with IPTG for 4 h, harvesting the cells, and analyzing the total cell protein by SDS–PAGE. Following protein detection at the desired M_r range, the corresponding DNA constructs were confirmed by Sanger sequencing (M13mp18 subclones).

DNA Sequence Determination. As shown in Figure 1, all *dadB* gene constructs possess terminal 5′-*Eco*RI and 3′-*Hin*dIII restriction sites. Each of the expression vectors was

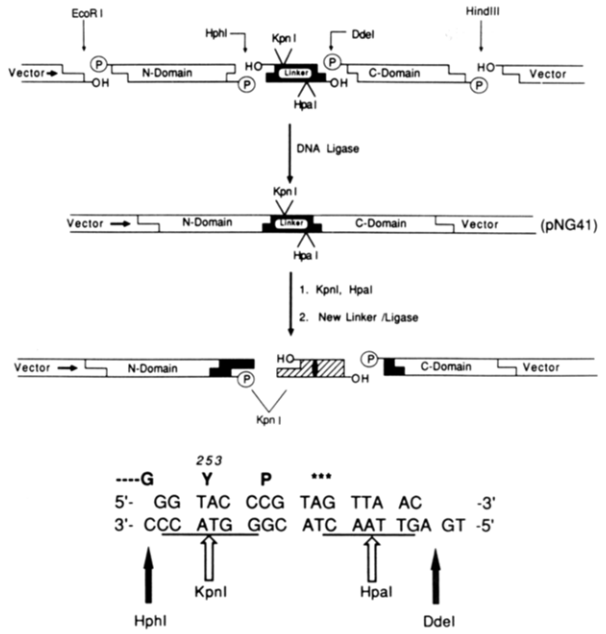

FIGURE 1: Schematic representation of the four-part ligation employed in the generation of the plasmid template for cassette mutagenesis (pNG41). The arrangement of a single phosphorylated 5′-end per ligation site was required for correct assembly. Also shown is the DNA and protein sequence of the linker that bears the unique *Kpn*I and *Hpa*I sites and an in-frame termination codon and possesses ends that are 5′-*Hph*I and 3′-*Dde*I compatible.

digested with *Eco*RI and *Hin*dIII, and the 1.4-kb fragment was isolated. This was then subcloned into M13mp18, and the linker region was sequenced by the Sanger method using the 3′-proximal primer (−30 b) described in Table I.

Protein Expression and Purification. All the plasmid constructs in this series were transformed into *E. coli* strain W3110 lac I^Q and grown at 37 °C to ca. OD_{600} = 0.7 in LB medium (0.5 L) supplemented with ampicillin at 100 mg/L. Protein expression was induced by the addition of IPTG (2 mM final concentration) and pyridoxine hydrochloride (50 μM final concentration), and the culture was incubated for an additional 6 h. The cells were harvested by centrifugation and chilled to 0 °C, and the cell paste (3 g) was washed with chilled 50 mM potassium phosphate buffer (35 mL, pH 7.2). All subsequent operations were performed at 4 °C unless otherwise stated. Potassium phosphate buffer (20 mM, pH 7.2) containing 2×10^{-5} M PLP, 0.01% mercaptoethanol, and 0.5 mM EDTA was used as the standard buffer (Esaki & Walsh, 1986).

The cells were resuspended in the standard buffer (15 mL) and lysed by French press at 14 000 psi. The lysate was then transferred to an ultracentrifuge tube and spun at 40 000 rpm for 2 h. The supernatant was recovered and directly applied to a DEAE-Sephacel column (1.5 × 11 cm) equilibrated with standard buffer at 15 mL/h. The column was washed successively with 48 mL of standard buffer, 38 mL of standard buffer supplemented with 45 mM NaCl, and finally 20 mL of standard buffer supplemented with 70 mM NaCl. The activity was eluted with standard buffer containing 95 mM NaCl (150 mL), and 5-mL fractions were collected. The active fractions were pooled, concentrated with an Amicon PM-10 membrane to 4 mL, and applied to an Ultrogel AcA-54 column (1.25 × 110 cm) equilibrated with standard buffer. The flow rate was controlled at 18 mL/h, and 5-mL fractions were collected. The representative elution and SDS–PAGE profiles for native DadB from *E. coli* W3110/pNG21 are shown in Figure 2.

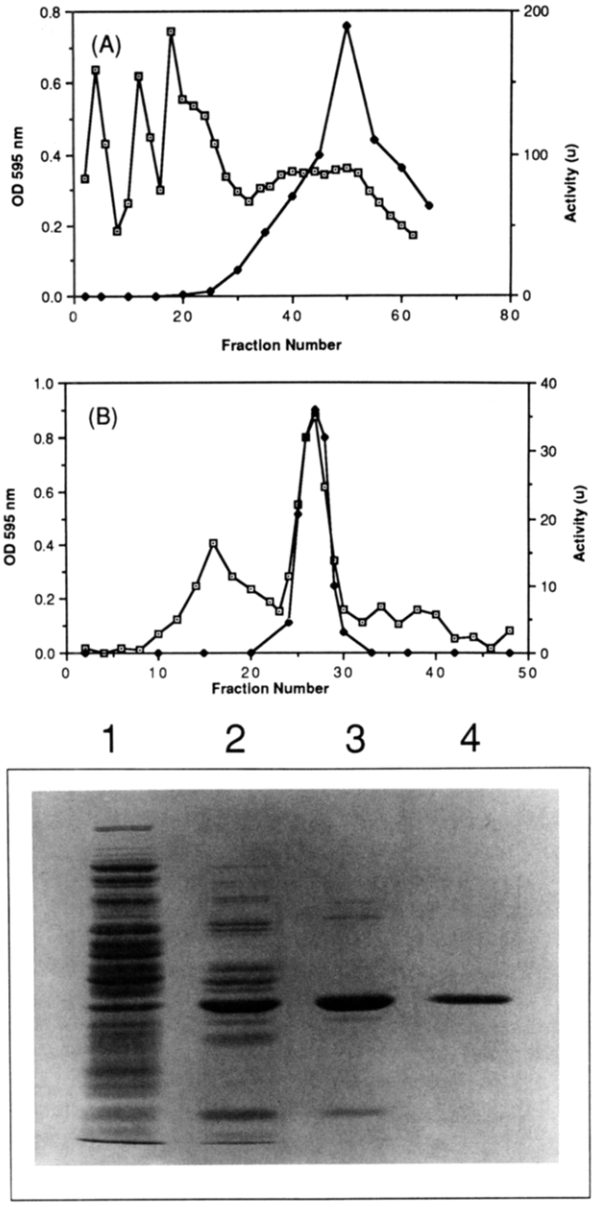

FIGURE 2: Representative elution and SDS–PAGE profiles of the purification steps followed for native and mutant DadB alanine racemases. Elution profile of native DadB was obtained from *E. coli* strain W3110/pNG21 during DEAE-Sephacel (A) and Ultrogel AcA-54 (B) chromatography: (⊡) OD at 595 nm; (■) units of activity. The active fractions from each column were pooled and analyzed by SDS–PAGE: (lane 1) crude cell lysate; (lane 2) active DEAE-Sephacel fractions; (lane 3) active AcA-54 fractions; (lane 4) DadB purified as in Wasserman et al. (1984).

Activity Assays. The V_{max} and K_m values for L-alanine were determined in the L- to D-Ala direction and quantitated by monitoring the decrease in absorbance at 340 nm of a mixture (1 mL) containing 100 mM Tricine/NaOH (pH 8.5), 50 mM L-alanine, 0.12 mM NADH, 110 units of lactate dehydrogenase, 1 unit of D-amino-acid oxidase, and racemase at 30 °C. A unit of alanine racemase is defined as the amount of enzyme that catalyzes the formation of 1 μmol of L- (or D-)alanine/min (Esaki & Walsh, 1986).

Protein Assays. Protein concentrations were determined either by the method of Lowry (Lowry et al., 1951) or by the procedure of Bradford (1976). Elution profiles of columns were constructed by measuring the absorption at 595 nm of samples containing protein at 2–50 μg/mL.

Spectrophotometric Determinations. All UV–visible spectra were recorded either on a Perkin-Elmer Model 554 or on a

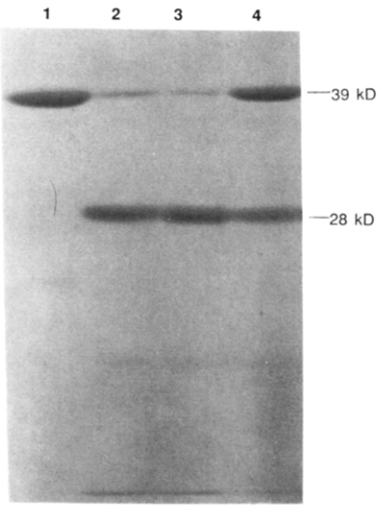

FIGURE 3: Limited proteolysis of DadB with subtilisin (0.5% w/w) at 30 °C for 4 h. Shown is an SDS–PAGE analysis of digest when the reaction was performed in the absence of substrate (lane 2) or in the presence of 100 mM L-Leu (lane 3) or 100 mM L-Ala (lane 4). Lane 1 corresponds to undigested DadB standard.

Perkin-Elmer λ 3 or a Hewlett-Packard diode array spectrophotometer. Circular dichroism spectra were taken in 1-mm cells at 4 °C by using an AVIV Model 60DS spectropolarimeter. The data obtained from eight repetitive scans at 0.2-nm bandwidth were averaged and scaled by using the instrument's computer.

Protein Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis (PAGE) was performed in the presence of SDS according to the method of Laemmli (1970). Usually the gels were stained with Coomassie brilliant blue R-250.

Limited Proteolysis. The effects of substrate-induced conformational change on native DadB were followed by proteolysis as described previously (Galakatos & Walsh, 1987). Quantitation of product formation was performed by scanning laser densitometry on Coomassie blue stained SDS gels and was based on the intensities of the 39-kDa native band (Figure 3). For the mutant proteins, a solution of the purified racemase (10 μg) in proteolysis buffer (42 μL; 50 mM Tris-HCl, pH 7.7, containing 2 mM EDTA, 5 mM mercaptoethanol, and 2×10^{-5} PLP) was treated with α-chymotrypsin (0.2 μg; 2% w/w) or subtilisin (0.05 μg; 0.5% w/w). Following incubation at 30 °C for 4 h, the reaction was quenched with 5 μL of 2 mM PMSF, and then 30 μL of Laemmli loading buffer was added. Samples were then analyzed by SDS–PAGE.

RESULTS

Substrate-Induced Conformational Changes. (*A*) *Limited Proteolysis.* The native DadB alanine racemase (39 kD) is cleaved by the nonspecific protease subtilisin to generate an N-terminal 28-kDa and a C-terminal 11-kDa fragment with net removal of a flexible tetrapeptide region 254–257 (Galakatos & Walsh, 1987). Figure 3 demonstrates the effect of a saturating concentration of substrate ($K_{m,L\text{-}Ala}$ = 8.2 mM; Wasserman et al., 1984)) on the rate of the proteolytic reaction followed by SDS–PAGE and quantitated by laser densitometry. Incubation of the racemase at 5 μM with 0.5% subtilisin at 30 °C for 4 h resulted in 97% proteolysis. A similar observation was made when the reaction was performed in the presence of 60 mM L-Leu, which has been shown to be inactive as a substrate (N. Galakatos, unpublished results). However, addition of 60 mM L-Ala in the reaction buffer and proteolysis under identical conditions resulted in only 26% conversion.

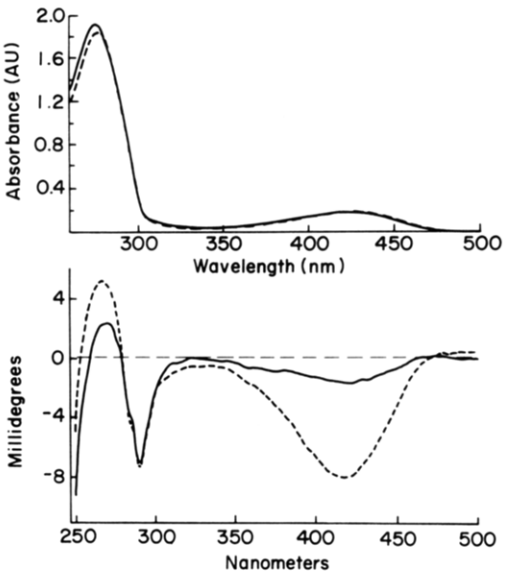

FIGURE 4: UV–vis and circular dichroism spectra of native DadB alanine racemase in standard buffer at 17 μM concentration. Spectra were recorded in the presence (solid trace) or absence (dotted trace) of 100 mM L-Ala and then superimposed computationally.

(*B*) *Circular Dichroism.* Figure 4 shows the UV–vis and CD spectra of DadB at 17 μM concentration in the standard buffer. When the buffer was supplemented with 100 mM L-Ala, significant Cotton effects were observed at the aromatic and PLP-absorbing (415 nm) regions, whereas UV–vis absorption remained unaffected by the addition of substrate.

Construction of pNG41 as a Template for Cassette Mutagenesis. Of the available site-directed mutagenesis methodologies, the cassette approach is most suitable when a given short sequence needs to be extensively altered in a nonrandom fashion. This was indeed the case for the desired hinge mutants. To utilize this technique, two proximal and unique restriction sites needed to be engineered around the DNA sequence encoding the hinge peptapeptide (Wells et al., 1985). For the *dadB* expression plasmid pNG21, no single silent mutations could be readily identified that would generate such an assembly. For this reason, a semisynthetic strategy was designed that involved the isolation of suitable N- and C-terminal encoding gene fragments, the synthesis of a linker bearing the unique *Kpn*I and *Hpa*I recognition sequences, and finally a triple-insert ligation into the original pNG21 vector.

Inspection of the restriction map of the *dadB* expression plasmid pNG21 revealed the presence of an *Hph*I and a *Dde*I site at positions encoding V^{251} and Q^{261}, respectively, which surround the -Y^{253}GGGY^{257}- region of interest. However, pNG21 contains 18 additional *Hph*I and 21 *Dde*I sites. To isolate the gene fragments encoding for the N- and C-terminal domains, the 1.4-kb *Eco*RI–*Hin*dIII gene cassette was first isolated and then treated in separate reactions with *Hph*I and *Dde*I. Although a partial digest was required in the former case, both fragments were readily isolated. In parallel, a synthetic linker was prepared that possessed 5′-*Hph*I and 3′-*Dde*I overhangs and the *Kpn*I and *Hpa*I recognition sequences and which upon ligation to the N-terminal encoding gene fragment would generate an in-frame termination codon at position 255 (Figure 1).

Although double-insert ligations are not uncommon (Mandecki et al., 1985), multiple-fragment condensations often suffer from polymeric insertion problems. When this four-part ligation was attempted with dephosphorylated vector and fully phosphorylated inserts, no desired product could be detected in all 24 transformants screened. However, when the same

Table II: Purification of DadB from *E. coli* W3110/pNG21[a]

purification step	activity (units)	protein[b] (mg)	units/mg	yield (%)
crude lysate	5412	132.0	41	100
DEAE-Sephacel	2522	6.6	382	47
Ultrogel AcA-54	1900	2.6	730	35

[a] 500-mL culture grown in LB medium at 37 °C and induced with IPTG for 6 h. [b] Protein concentration determined by the Bradford method (Bradford, 1976).

ligation was performed with unphosphorylated linker, greater than 85% of the plasmids assayed possessed the correct sequence for pNG41. As depicted in Figure 1, the latter strategy positions a single 5′-posphate functionality per ligation site.

Construction of Cassette Mutants. Plasmid pNG41 fulfills all the requirements for cassette mutagenesis at the hinge region of DadB by means of the unique and proximal nature of the *Kpn*I and *Hpa*I restriction sites. To generate the desired mutants, oligonucleotide cassettes encoding the mutations and possessing 5′-*Kpn*I and 3′-blunt ends were synthesized and then directionally inserted into pNG41 that had been digested with *Kpn*I and *Hpa*I (Figure 1). Since intact pNG41 would yield a C-terminal truncated form of the protein, initial screening of transformants was easily performed by direct expression and SDS–PAGE analysis of total cell protein. Once promising transformants were identified by this method, their DNA sequence was confirmed by subcloning the gene cassette into M13mp18 followed by Sanger sequencing.

Protein Expression and Purification. Earlier work from these laboratories demonstrated the cloning and initial expression of DadB (Wasserman et al., 1983). When the *dadB* gene was subcloned behind the tac promoter (pNG21) and expressed in *E. coli* strain W3110 lac I^Q by IPTG induction, the native racemase was produced at 10 mg/L of culture (4.5% of soluble protein). This represents an 140-fold improvement over the previously reported system.

Because of the high levels of DadB generated by the *E. coli* W3110/pNG21 expression system and the need to purify the mutant proteins rapidly, the original purification scheme (Wasserman et al., 1984) was successfully reduced to a two-column procedure that takes only 2 days to complete and does not involve any dialysis steps. As shown in Table II and Figure 2 for native DadB, this short procedure did not compromise purity or specific activity. For the Ultrogel-purified protein, laser densitometry on the SDS gel indicated 96% purity.

E. coli is known to possess its own system of two alanine racemases (Wild et al., 1985). Since *E. coli* strain W3110 encodes an endogenous pair of Ala racemases, the level of background activity was estimated by IPTG induction of the parent expression vector pKK223-3 (Pharmacia) in W3110. In crude cell lysates, this control activity was <0.4% of native DadB expressed under identical conditions from W3110/pNG21.

The mutant proteins were also expressed and purified as described above, and yielded 1–2 mg of enzyme/500 mL of culture. However, in the case of the W3110/pNG421 (Δ Y^{253}GGG) and W3110/pNG41 (Δ C-terminal domain) deletion mutants, no protein of anticipated M_r could be detected by SDS–PAGE. Although the possibility of incomplete transcription cannot be excluded, this result can be rationalized in terms of instability of the translation product and subsequent digest by cytoplasmic proteases.

Kinetic Characterization of Mutant Racemases. The K_m and V_{max} values for the wild-type and mutant proteins purified as described above were calculated from double-reciprocal plots by using data obtained for the conversion of L- to D-Ala by the spectrophotometric assay described under Materials and Methods (Table III).

Table III: Catalytic Efficiency of DadB Hinge Mutants

hinge sequence	plasmid construct	K_m[a] (mM)	V_{max}/K_m (%)
-Y^{253}GGGY^{257}-[b]	pNG21	6.8	100
-Y^{253}GGY^{256}-	pNG422	5.9	102
-Y^{253}GY^{255}-	pNG423	6.8	105
-Y^{253}GGGGY^{258}-	pNG424	8.7	21
-Y^{253}PGGY^{257}-	pNG460	5.4	60
-Y^{253}GPGY^{257}-	pNG440	4.8	126
-Y^{253}GGPY^{257}-	pNG450	8.1	26

[a] For L-Ala. [b] Native DadB hinge sequence.

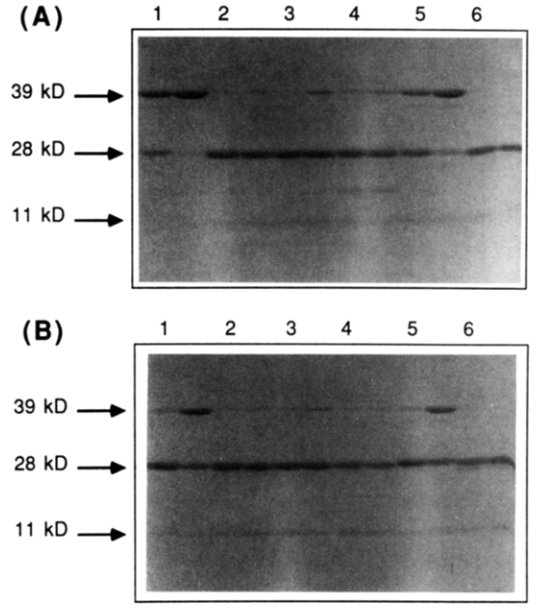

FIGURE 5: (A) Limited proteolysis of native DadB and mutants with α-chymotrypsin (2% w/w) at 30 °C for 4 h. (B) Subtilisin digest (0.5% w/w) under the same conditions. The lanes to the right of each numbered entry denote that the proteolytic reaction was performed in the presence of 60 mM L-Ala. (Lane 1) Native DadB; (lane 2) (–G); (lane 3) (–GG); (lane 4) (+G); (lane 5) G255P; (lane 6) G254P.

Limited Proteolysis on Hinge Mutants. Both the native and mutant alanine racemases purified as described above were subjected to limited proteolysis with α-chymotrypsin (2% w/w) and subtilisin (0.5% w/w) in the presence or absence of 60 mM L-Ala (Figure 5). In all cases the proteolytic reaction was terminated by the addition of PMSF, and the products were analyzed by SDS–PAGE. As observed earlier (Galakatos & Walsh, 1987), both proteases generated two fragments (28 and 11 kDa), and the rate of chymotryptic digestion under these conditions was slower than that with subtilisin. This was also the case for all the mutant proteins. Moreover, the reduction in proteolytic rates by the presence of a saturating concentration of substrate observed for native DadB was also conserved in all the mutants. The differential chymotryptic activities allowed the qualitative determination of protease sensitivity of the hinge mutants (in order of decreasing stability) native ≈ G255P ≫ (Δ GG) > (Δ G) > (+G) > G254P, G256P.

Discussion

Earlier studies on the specific proteolysis of the isoenzymic DadB and Air alanine racemases from *S. typhimurium* demonstrated that chymotrypsin and subtilisin selectively recognize and cleave at the peptide -Y^{253}GG(G or T)(Y or W)^{257}- (Galakatos & Walsh, 1987). Secondary structure and hy-

Table IV: Primary Sequence Alignment of Alanine Racemases

racemase	primary sequence at loop region													
DadB[a]	G^{248}	E	R	V	G	Y	G	G	G	Y	S	V	T	Q	E^{262}
Alr[a]	G^{250}	E	P	V	G	Y	G	G	T	W	V	S	E	R	D^{264}
Bdal[b]	G^{259}	E	K	V	S	Y	G	A	T	Y	T	A	Q	T	E^{273}
Sdal[c]	G^{261}	E	S	V	S	Y	G	A	E	Y	T	A	E	K	D^{275}

[a] From *S. typhimurium*. [b] From *B. stearothermophilus*. [c] From *B. subtilis*.

drophilicity predictions support the intuitive assumption that this flexible hinge region lies on the protein surface and indicate a clear structural motif transition from α/β to β, centered at the hinge region (T. Webster and N. Galakatos, unpublished results).

A striking prediction, however, is that the hinge peptide is part of a larger proposed loop region (residues 248–262 for DadB) that is conserved in both size and amino acid composition (Table IV). This is unusual since comparison of homologous proteins of known structure reveals that most insertions and deletions as well as radical sequence changes occur in loops (Thorton et al., 1988). Sequence conservation may imply functional constraints on this region for enzyme activity.

Alanine racemases are not unique among PLP enzymes in possessing such a structural feature. A glycine-rich region of equivalent primary sequence locus has recently been identified in the monomeric PLP enzyme D-serine dehydrase and implicated in the binding of the cofactor (Marceau et al., 1988a). This polyglycine pattern is present in eight additional PLP-requiring enzyme classes (Marceau et al., 1988b).

Studies on the clipped two-domain DadB protein demonstrated that removal of the -GGGY- hinge did not significantly affect the active-site environment as assessed by the incorporation of 0.76 equiv of the mechanism-based inactivator β-chloro-D-alanine but caused a 30-fold reduction in apparent V_{max}. Moreover, proteolysis did not perturb secondary structure (UV–CD spectra unchanged), and the two fragments remained associated under native conditions, presumably due to extensive interdomain hydrophobic contacts (Galakatos & Walsh, 1987). On the basis of these results the role of the hinge region has now been examined here, both as a possible required pivot area for domain motion (Huber, 1988) and as a region that may directly interact with the active site. In a series of experiments not discussed in the present paper, the hinge sequence was also selected as a docking region for the engineering of chimeric alanine racemases from Gram-negative and Gram-positive organisms (N. Galakatos, unpublished results).

Initial experiments in the present study demonstrated the existence of at least two distinct conformational states for DadB that depend on the presence of substrate. In the first case, a substrate-induced change in hinge conformation was readily detected by the reduction in rate of the regiospecific proteolytic reaction. In parallel, active-site geometry perturbations were observed by circular dichroism. The fact that a significant Cotton effect at 415 nm was observed upon addition of 100 mM L-alanine that was not accompanied by any changes in UV absorption suggests that the presence of substrate is responsible for a reorientation of the PLP moiety at the active-site cleft.

The requirement for conformational reorganization at the active site to accommodate substrate can be rationalized in terms of the mechanism of the racemization reaction. After conversion of resting PLP=NH-Lys^{35} aldimine to L- or D-Ala=PLP aldimine with concurrent release of ϵ-NH_2-Lys^{35}, the latter amino functionality probably acts as a catalytic base to abstract the alanine α-hydrogen to yield a planar, fully conjugated anionic PLP–substrate intermediate complex. Reprotonation can yield either D-Ala=PLP or L-Ala=PLP aldimines from which transaldimination once again with ϵ-NH_2-Lys^{35} regenerates resting enzyme and releases product (Faraci & Walsh, 1988). The two protein conformational isomers suggested by the CD and proteolytic studies may reflect the distinct planar geometries required for the two ligation states of the cofactor.

Since crystallographic data are not yet available for PLP-containing alanine racemases (Neinhart et al., 1987), this argument can be further supported by analogy to the free and inhibitor-bound crystal forms of the PLP-requiring mitochondrial aspartate aminotransferase from chicken heart (AAT). Cocrystallization of AAT with the inhibitor 2-methylaspartate resulted in rigid-body movement that was accompanied by a 30° rotation of the pyridine ring of PLP (Jansonius et al., 1984). Such domain "closure" was also followed by the addition of substrate or inhibitor in proteolytic (Sandmeier & Christen, 1980), deuterium exchange (Pfister et al., 1985), and circular dichroism experiments (Martinez-Carrion et al., 1978).

The detection of two protein conformers and the maintenance of catalytically active clipped DadB following proteolysis led us to the hypothesis that upon substrate binding the DadB protein may also undergo domain closure and this structural reorganization is transduced to the hinge peptide which then undergoes a conformational change. Similar syncatalytic processes have been postulated for an array of two-domain enzymes such as citrate synthetase (Wiegand & Remington, 1986), 3-phosphoglycerate kinase (Adams et al., 1985), and hexokinase (Huber, 1988). In most cases, hinge regions have been implicated as "pivot" regions serving as rotational axes for domain movement. However, although the functional significance of protein flexibility and mechanism of domain closure has been well recognized (Huber & Bennett, 1983; Lesk & Chothia, 1984), only recently has this problem been addressed experimentally. For phosphoglycerate kinase, the interdomain hinge was successfully employed as a transfer region for chimeric enzyme engineering (Mas et al., 1986), and site-directed mutations on hinge residue E190 were prepared to probe effects on catalytic activity (Mas et al., 1987). E190Q and E190D mutations demonstrated that this residue, which is distant from the active site, is not directly involved in substrate binding. These substitutions caused a maximal 17-fold drop in V_{max}, which is presumably attributable to constraints imposed on the conformational flexibility.

For DadB, the effects of hinge length and conformational constraint on catalytic efficiency have been evaluated. To facilitate this study, the *dadB* gene was subcloned behind the strong tac promoter and expressed in *E. coli* by IPTG induction as 4.5% of cell protein. A plasmid serving as a template for cassette mutagenesis was generated and was later employed in the construction of eight mutant genes. In addition, a rapid purification procedure was devised that allowed the isolation of 1–2 mg of pure protein/500 mL of culture.

Once the native DadB enzyme protein has folded, the full hinge sequence ($G^{254}GGY^{257}$) can be proteolytically removed, and the clipped protein retains 3% of V_{max} activity (Galakatos & Walsh, 1987). To explore the hypothesis that the proteolytically defined N-terminal domain (residues 1–254) of DadB also meets the criterion of "independent folding" (Richardson, 1981), an in-frame termination codon was introduced at position 255. Upon induction of the gene construct, the desired protein (M_r *ca.* 28 000) could not be detected by SDS–PAGE.

Similarly, a mutant encoding a full hinge-deleted polypeptide (Δ GGGY) failed to produce the expected protein. If indeed extensive nonbonded interactions are required for protein stabilization as the proteolytic results suggest, then it is quite possible that the hydrophobic interdomain contact area of the C-terminal fragment is also needed for N-terminal domain folding. Conversely, if the interdomain linear sequence is severely truncated, then the nonbonded interactions may severely hamper the folding process yielding an unstable protein. Since genetically engineered deletion of the hinge was completely deleterious, we examined mutants possessing altered hinge length and conformational flexibility.

Insertion of a fourth glycine residue to the -YGGGY- hinge peptide generated an enzyme species (-YGGGGY-) 5-fold less efficient than the native protein. The additional degrees of freedom thus introduced may affect the optimal alignment of the two domains. Since the effect was primarily on V_{max}, it is likely that the hinge region is not directly involved in substrate binding. This postulate is supported by all the other mutagenesis data presently reported and the earlier results on proteolytic hinge removal from the native protein (Galakatos & Walsh, 1987).

When mutants encoding the deletion of one glycine (-YGGY-) and then two glycine residues (-YGY-) were generated, it was surprising that full retention of catalytic activity was observed. This result clearly demonstrates the nonessential role of these two positions and indicates that only a tripeptide connecting domain is required for the enzyme protein to fold to a structure of full activity. It is possible that the hinge-containing loop of DadB (residues 248–262) adopts an ω conformation (Lesxczynski & Rose, 1986) and the catalytically important packing of such structure is not perturbed by these deletions.

In another series of experiments the degrees of freedom at each of the glycine positions was restricted by Gly to Pro substitutions. These point mutants also had no effect on L-Ala substrate K_m and thus confirm the earlier conclusion that the hinge glycines are not involved in substrate binding. Conformational constraint due to G254P or G256P resulted in moderate V_{max} reductions, whereas the G255P mutation actually yielded a slightly improved enzymatic species. In the absence of more biophysical and structural data these results cannot be conclusively interpreted. However, it is important to recall that in the extreme case of proteolytic removal of the hinge tetrapeptide a 30-fold decrease in V_{max} was observed.

Proteolytic susceptibility experiments on these hinge mutant enzymes in the presence or absence of substrate confirmed the conformational isomerization effect also observed for native DadB. Although individual proteolytic rates could not be directly correlated with enzymatic efficiency, their differential protease sensitivity can be interpreted to reflect the individual conformational preferences of mutant hinge sequences adopted in their interaction with the protease.

In conclusion, our studies on the proteolytically defined hinge region (Y^{253}–Y^{257}) of the DadB alanine racemase have focused on the design of a cassette mutagenesis strategy to generate mutations that alter both the length and sequence of that pentapeptide region. Given that neither the N-terminal 1–255 fragment nor the hingeless but otherwise full-length enzyme gives detectable protein, it is likely that the hinge region is a crucial element in the folding of the alanine racemase into a compact and correctly positioned two-domain enzyme. Tri- and tetrapeptide hinge lengths are fully viable, while a hexapeptide hinge results in an enzyme species of 5-fold decreased V_{max}, suggesting the incorporation of excess flexibility in the loop. The effect of proline substitutions at any of the three glycine loci (254–256) was again quite modest, indicating that the local structure of the loop in the mature, folded enzyme may not be critical for activity and that loop proline substitutions do not substantially affect proper folding. None of the mutations appears to interfere with substrate K_m or PLP binding (data not shown), suggesting that this glycine-rich loop is not functioning in a polyglycine PLP-binding motif recently proposed for D-serine dehydratase (Marceau et al., 1988).

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Salmonella typhimurium Histidinol Dehydrogenase: Complete Reaction Stereochemistry and Active Site Mapping†

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ABSTRACT: The stereochemistry of the L-histidinol dehydrogenase reaction was determined to be *R* at NAD for both steps, confirming previous results with a fungal extract [Davies, D., Teixeira, A., & Kenworthy, P. (1972) *Biochem. J. 127*, 335–343]. NMR analysis of monodeuteriohistidinols produced by histidinol/NADH exchange reactions arising via reversal of the alcohol oxidation reaction indicated a single stereochemistry at histidinol for that step. Comparison of vicinal coupling values of the exchange products with those of L-alaninol and a series of (*S*)-2-amino-1-alcohols allowed identification of the absolute stereochemistry of monodeuteriohistidinols and showed that histidinol dehydrogenase removes first the *pro-S* then the *pro-R* hydrogens of substrate histidinol. The enzyme stereochemistry was confirmed by isotope effects for monodeuteriohistidinols as substrates for the *pro-R*-specific dehydrogenation catalyzed by liver alcohol dehydrogenase. Active site mapping was undertaken to investigate substrate–protein interactions elsewhere in the histidinol binding site. Critical binding regions are the side-chain amino group and the imidazole ring, whose methylation at the 1- or 2-position caused severe decreases in binding affinity. Use of alternative substrates further clarified active site interactions with the substrate. Compounds in which the α-amino group was replaced by chloro, bromo, or hydrogen substituents were not substrates of the overall reaction at 1/10 000 the normal rate. The α-hydroxy analogue of histidinol was a substrate, with $k_{cat}/K_m = 0.1\%$ that of histidinol. Removal or alteration of the imidazole ring also prevented catalysis, resulting in complete inactivity with alaninol or thienylalaninol.

Salmonella typhimurium histidinol dehydrogenase (EC 1.1.1.23) catalyzes the four-electron dehydrogenation of L-histidinol to L-histidine using 2 mol of NAD, probably at a single active site (Adams, 1955). The enzyme is one of three known NAD-linked four-electron dehydrogenases [the two others are UDP-glucose dehydrogenase (EC 1.1.1.22) and 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34)]. Such enzymes are interesting mechanistically because they oxidize both a substrate alcohol and an intermediate aldehyde, reactions with quite different redox potentials. In the case of HMGR[1] the substrate is a thiol ester, and a cysteine–UDP glucuronic thiol ester is believed to be an intermediate for UDPGDH (Ridley et al., 1975). Such a thiohemiacetal–thiol ester oxidation is much closer in redox potential to the alcohol–aldehyde reaction. An active site cysteine has recently been identified in HDH (Grubmeyer & Gray, 1986), suggesting that a thiol ester may be involved in this enzyme as well.

To learn how HDH catalyzes two sequential oxidations, we have sought to understand the structure of the active site. Stereochemical studies on NADH produced by the HDH activity of a crude extract of *Neurospora* have been performed previously (Davies et al., 1972) and showed that the reaction was *R*(A) for both steps [UDPGDH is *S*(B) for both steps, and HMGR is *R*(A) for both steps; You, 1982]. However, HDH from yeast is known to be a multifunctional enzyme (Donahue et al., 1982), and the recent finding of opposite stereochemistry at NADH for liver and *Drosophila* ADH (Benner et al., 1985) suggested that additional stereochemical studies on HDH from *Salmonella* were important. We have also been able to use exchange reactions, NMR analysis, and isotope effects to determine the stereochemistry of the sequential hydrogen removals from histidinol, and thus to orient the coenzyme with respect to the substrate molecule in the first transition state, and with the hypothesized thiohemiacetal in the second transition state. Finally, we have also investigated the origins of the high substrate specificity of the histidinol dehydrogenase reaction by using histidinol analogues as competitive inhibitors and alternative substrates to map the functionally important binding interactions between substrate and enzyme.

Materials and Methods

Materials. L-Histidinol, L-histidine, D-histidine, L-alaninol, histidine methyl ester, L-histidine hydroxamate, histamine, 2-methylimidazole, L-β-imidazol-4-yllactic acid, DL-triazol-

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[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDH, histidinol dehydrogenase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LADH, liver alcohol dehydrogenase; SMP, submitochondrial particles; UDPGDH, uridine diphosphoglucose dehydrogenase; YADH, yeast alcohol dehydrogenase. For terminology regarding labeling of histidinol, see Materials and Methods.

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