Specific Proteolysis of Native Alanine Racemases from *Salmonella typhimurium*: Identification of the Cleavage Site and Characterization of the Clipped Two-Domain Proteins†

Nicholas G. Galakatos and Christopher T. Walsh*

Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: Native DadB and Alr alanine racemases (M_r 39 000) from *Salmonella typhimurium* are proteolyzed at homologous positions by α-chymotrypsin, trypsin, and subtilisin to generate in all cases two nonoverlapping polypeptides of M_r 28 000 and 11 000. Under nondenaturing conditions, chymotryptic digest results in an associated form of the two fragments which possesses 3% of the original catalytic activity, incorporates 0.76 equiv of the mechanism-based inactivator β-chloro-[^{14}C]-D-alanine [Badet, B., Roise, D., & Walsh, C. T. (1984) *Biochemistry 23*, 5188], and exhibits a UV circular dichroism profile identical with that of native enzyme. Protein sequence analysis of the denatured chymotryptic fragments indicates the presence of a tetrapeptide interdomain hinge (DadB, residues 254–257; Alr, residues 256–259) that is attacked at both ends during proteolysis. Under the previously employed digest conditions, NaB^{3}H$_4$-reduced DadB holoenzyme is resistant to α-chymotrypsin and trypsin and is labile only toward subtilisin. These data suggest that the hinge structure is essential for a catalytically efficient enzyme species and is sensitive to active site geometry. The sequence at the hinge region is also conserved in alanine racemases from Gram-positive bacteria.

In *Salmonella typhimurium* the conversion of L-alanine to D-alanine is catalyzed by two isoenzymes, DadB and Alr (Wasserman et al., 1983). The DadB and Alr alanine racemases are monomeric proteins of similar size (39 000), possess extensive overall sequence identity (43%), and in their active form bind 1 mol of pyridoxal 5′-phosphate (PLP) cofactor at equivalently positioned lysyl residues near the N-terminus (Galakatos et al., 1986). However, the active site environments of DadB and Alr appear to be distinctly different: DadB catalyzes the racemization of L-alanine 60 times faster than Alr; conversely, when inactivated with β-haloalanines, the partition ratio of inactivation to turnover for DadB is fivefold lower than that for Alr (Esaki & Walsh, 1986). More importantly, the respective racemase-encoding genes map distinctly apart on the *S. typhimurium* chromosome (*dadB*, minute 37; *alr*, minute 91) and appear to be differentially regulated (Daub et al., 1987). In an effort to study the effect of gene duplication on the conservation of protein tertiary structure and to investigate possible racemase structure subdivision into catalytic and regulatory domains, we undertook a study of the susceptibility of DadB and Alr toward controlled proteolysis.

Materials and Methods

Materials. L- and D-alanine, L-alanine dehydrogenase (EC 1.4.1.1) from *Bacillus subtilis* (30 units/mg), D-amino acid oxidase (EC 1.4.3.3) from hog kidney (15 units/mg), pyridoxal 5′-phosphate (PLP), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma. Hog muscle lactate dehydrogenase (EC 1.1.1.27) (550 units/mg), α-chymotrypsin, subtilisin, carboxypeptidase A, and carrier-fixed α_2-macroglobulin were obtained from Boehringer-Mannheim Biochemicals. TPCK-treated trypsin was from Cooper-Worthington Biochemicals. Ultrapure sodium dodecyl sulfate (SDS) and Coomassie brilliant blue R-250 were from BRL. Acrylamide (>99.9%) was from Bio-Rad. NaB^{3}H$_4$ (282 mCi/mmol) was purchased from New England Nuclear. β-Chloro-[^{14}C]-D-alanine (0.247 mCi/mmol) was a generous gift of Dr. B. Badet (Badet et al., 1984) of these laboratories. All other chemicals were of analytical reagent grade.

Enzymes. The DadB alanine racemase (900 units/mg) was purified from *Salmonella typhimurium* strain DB9071/pSW12 as previously reported (Wasserman et al., 1984) and was a kind gift of Dr. N. Esaki of these laboratories. The Alr alanine racemase (11 units/mg) was obtained in pure form from *Escherichia coli* strain W3110/pNG10, which produces the enzyme as 3.3% of the total cell protein (Galakatos and Walsh, unpublished results). The racemase from *Bacillus stearothermophilus* (Bsar) was provided by Dr. Badet of these laboratories (Inagaki et al., 1986).

Activity Assays. The production of L-alanine from D-alanine was determined by following the increase in absorbance at 340 nm of a reaction mixture (1 mL) containing 100 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine)/NaOH (pH 8.5), 0.15 unit of L-alanine dehydrogenase, 50 mM D-alanine, 10 mM NAD, and enzyme at 30 °C.

The generation of D-alanine from L-alanine was 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 enzyme 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 per minute (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 (Bradford, 1976). Elution profiles of columns were constructed by measuring the absorption at 595 nm of samples containing protein at 2–50 μg.

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

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

Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis (PAGE) was performed in the presence of SDS according to Laemmli (Laemmli, 1970). Usually the gels were stained with Coomassie brilliant blue R-250. ^{3}H- or ^{14}C-labeled protein fragments were detected by autoradiography of gels that had first been immersed in an enhancer solution (ENLIGHTENING, New England Nuclear) for 30 min and then dried at 60 °C in vacuo. For preparative SDS–PAGE, the procedure of Isenberg was employed (Spiker & Isenberg, 1983). The desired bands were excised from the gel, and the protein was electroeluted by the method of Hunkapiller (Hunkapiller et al., 1983).

Buffers. Buffer A was 20 mM KP_i, pH 7.2, containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1.4 mM 2-mercaptoethanol, and 2×10^{-5} M PLP. Buffer B was 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.7, containing 2 mM EDTA and 5 mM 2-mercaptoethanol. Buffer C was buffer B supplemented with 5×10^{-5} M PLP (Hogberg-Raibaud & Goldberg, 1977a). Buffer D was buffer C containing 100 mM NaCl.

Limited Proteolysis. A solution of the racemase (5.0 mg) in buffer C (4.0 mL) was treated with α-chymotrypsin (100 μg; 2% w/w) at 30 °C for 7 h. To the mixture was then added NaCl to a final concentration of 100 mM. The sample was then transferred to a vessel containing 1 mL of resin-bound α_2-macroglobulin gel (Porath et al., 1975) equilibrated with buffer D, and the resulting suspension was incubated at room temperature with gentle shaking for 30 min. The gel was then removed by centrifugation, and the supernatant was concentrated to ca. 1 mL in a nitrocellulose collodion bag (UH 100/10, Schleicher & Schuell Inc.). This sample was then passed through a Sephadex G-75 superfine column (1.5 × 65 cm) equilibrated in buffer C at 4 mL/min, 1.7-mL fractions being collected. A single protein-containing peak eluted in fractions 26–30. These were pooled and concentrated as described above. Analytical experiments with trypsin and subtilisin were performed as described above, except that at the end of the proteolytic reaction the mixture was quenched with PMSF to 0.2 mM and the samples were immediately analyzed by SDS–PAGE.

NaB^3H_4 Reduction of DadB and Localization of the Label on the Nicked Protein. To a solution of DadB (595 μg) in the PLP-containing buffer A (350 μL) was added solid NaB^3H_4 (ca. 0.1 mg). Upon being vortexed, the characteristic pale yellow color of the enzyme solution bleached (Badet et al., 1984). The resulting clear mixture was allowed to stand at 0 °C for 2 h and was then applied to a Sephadex G-25 column (1 × 21 cm) equilibrated with buffer B. The protein-containing fractions were pooled and then concentrated in a nitrocellulose collodion bag (UH 100/25, Schleicher & Schuell Inc.). Aliquots (40 μg) of this material were then treated in separate reactions with α-chymotrypsin, trypsin, and subtilisin under the conditions described under Limited Proteolysis. Following the 7-h incubation step, each reaction was stopped by addition of PMSF to 0.2 mM. The product mixtures were then resolved on a 12% analytical polyacrylamide gel containing SDS. The bands were visualized both by staining with Coomassie blue and by autoradiography.

Active Site Labeling of Nicked DadB. Radioactive labeling experiments were performed according to Badet (Badet et al., 1984). A solution of the proteolyzed racemase (1.42 mg) in buffer C (1.9 mL) was placed in a 3-mL cuvette and equilibrated at 15 °C. To this was added β-chloro-[^{14}C]-D-alanine (100 μL) at 0.5 M, 0.247 mCi/mmol. The progress of the reaction was monitored by the drop in bound PLP absorbance at 415 nm and appeared to reach completion in 10 min. An additional 50-μL aliquot of the radioactive inactivator was then added, but no further change in absorbance was observed. After 10 min the mixture was cooled to 0 °C, and $NaBH_4$ (4 mg) was cautiously added. The resulting bleached solution was incubated at 4 °C for 45 min and then applied to a Sephadex G-75 superfine column. The product was eluted with buffer B at 4 mL/min, 1.2-mL fractions being collected. The protein-containing peak eluted cleanly ahead of small labeled materials. The specific radioactivity of the protein was determined.

NH_2-Terminal Sequence Determination. The polypeptide fragments generated from the limited proteolysis of DadB and Alr were separated on 12% preparative SDS–PAGE and then electroeluted as described above. Edman degradation on these samples was performed by Drs. D. Andrews and W. Lane at the Harvard Microanalysis Facility, Cambridge, MA, using an Applied Biosystems Model 470A automated sequencer. The PTH derivatives obtained were analyzed on a Hewlett-Packard Model 1090 HPLC system equipped with a 1040 Diode Array detector.

COOH-Terminal Sequence Analysis. To a solution of the electroeluted proteolytic polypeptide fragment (ca. 1 nmol) in 100 mM *N*-ethylmorpholine/acetic acid buffer of pH 8.1 (100 μL) was added carboxypeptidase A (10 μL of suspension). The mixture was then incubated at room temperature for 8 h, and the sample was lyophilized. Two cycles of dissolution, freezing, and lyophilization were undertaken in the "coupling" buffer of ethanol–H_2O–triethylamine (7:1:1). The final product was then dissolved in coupling buffer (90 μL), phenyl isothiocyanate (10 μL) was added, and the mixture was allowed to react at room temperature for 20 min. The product was then frozen and lyophilized. The resulting sample was dissolved in 0.14 M NaOAc buffer of pH 6.4 (100 μL) and was analyzed on a Hewlett-Packard Model 1084B HPLC system equipped with an Altex-ODS column.

Homology Searches. Protein sequence homology searches were performed with the National Biomedical Research Foundation PSQ data bank and through the kind collaboration of Professor R. Doolittle at U.C. San Diego.

RESULTS

Limited Proteolysis. Alanine racemases DadB and Alr from *S. typhimurium* were subjected to controlled proteolysis by two residue-specific endopeptidases (α-chymotrypsin and trypsin) and the nonspecific protease subtilisin. Each of the six digests was monitored by both SDS–PAGE and catalytic activity.

As shown in Figure 1, proteolysis of the catabolic alanine racemase DadB with α-chymotrypsin generates two fragments of sizes 27 and 11 kDa and results in a time-dependent loss of catalytic activity that follows apparent first-order kinetics. At the end of a 7-h incubation period, proteolyzed DadB appears to retain partial activity at ca. 3% of the native enzyme.

Similar-sized fragments are generated when DadB is treated with trypsin and subtilisin (Table I). However, the rates of the proteolysis reactions appear to be dependent on the endopeptidase used: Overall, subtilisin digests DadB the fastest (reaction complete within 30 min) followed by α-chymotrypsin ($t_{1/2}$ = 0.8 h) and trypsin ($t_{1/2}$ = 5 h).

Table I: Limited Proteolysis of Alanine Racemases

alanine racemase			proteolytic fragments				
			determined sequence				
name	size (kDa)	protease[a]	NH_2 end	CO_2H end	size (kDa)	domain[b]	peptide released
DadB	39	α-chymotrypsin	M^1-T-R-P-I-	-V-G-V-Y^{253}	27.8	N	G^{254}-G-G-Y^{257}
			S^{258}-V-T-Q-E-		10.9	C	
		subtilisin	M^1-T-R-P-I-		27.8	N	
			S^{258}-V-T-Q-E-		[10.9	C_1][c]	
			Q^{261}-E-Q-R-I-		[10.6	C_2]	
		trypsin			27.3	N	(V^{251}-...-R^{264})[d]
					10.5	C	
Alr	39	α-chymotrypsin	M^1-Q-A-A-T-	-Y^{255}	28.0	N	G^{256}-G-T-W^{259}
			V^{260}-S-E-R-D-		10.8	C	
		subtilisin			28.0	N	
					10.7	C	
		trypsin			27.0	N	(A^{249}-...-R^{263})[d]
					10.5	C	
Bsar	39[e]	subtilisin			27.0	(N)	
					12.0	(C)	

[a] Protease specificities: α-chymotrypsin, Y, W, and F; subtilisin, nonspecific; trypsin, R and K. [b] N, amino domain; C, carboxyl domain. [c] Relative ratio C_1:C_2 = 1:2 based on V^{259}:I^{265} PTH derivative integration during Edman degradation. [d] Predicted sequence. [e] In denatured form, as a monomer.

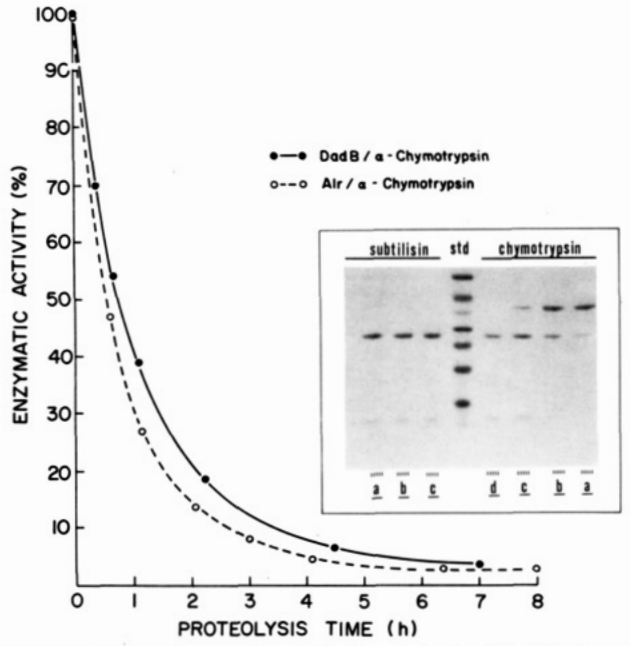

FIGURE 1: Time course of the limited proteolysis of the DadB and Alr alanine racemases from *S. typhimurium*. Progress of the digest of each racemase with α-chymotrypsin (2% w/w) in 50 mM Tris-HCl, pH 7.7, containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 5 × 10^{-5} M PLP was monitored by the loss of catalytic activity. Activity appears to level off at ca. 3% of the initial value. (Insert) SDS–PAGE analysis of reaction products of DadB with α-chymotrypsin and subtilisin under the same conditions as above. Aliquots removed from the mixture at the times listed below were quenched with PMSF to 0.2 mM and kept at −20 °C until analysis: (a) 0.5, (b) 1, (c) 3, and (d) 6 h. The std lane contains standards at M_r 66 000, 45 000, 36 000, 29 000, 24 000, 20 100, and 14 200.

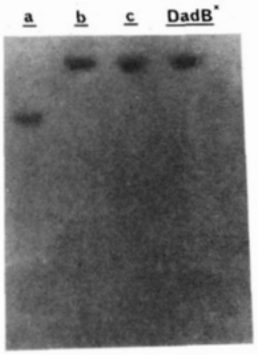

FIGURE 2: Proteolysis of NaB^3H_4-reduced DadB holoenzyme. SDS–PAGE radioactivity profile of reductively labeled DadB (DadB*) when treated with subtilisin (a), trypsin (b), and α-chymotrypsin (c). The band corresponding to the labeled NH_2-terminal domain (M_r 28 000) appears only in lane a.

The biosynthetic alanine racemase Alr closely parallels the proteolytic and activity profiles of DadB (Table I; Figure 1). In one experiment, the dimeric thermophilic alanine racemase Bsar from *Bacillus stearothermophilus* was subjected to controlled subtilisin digest. The fragmentation pattern observed resembles closely the ones obtained from the proteolysis of the equivalently sized but monomeric racemases from *S. typhimurium* (Table I).

Determination of the Proteolytic Cleavage Site. Since the PLP-binding lysyl residues of DadB and Alr are both located at the amino-proximal 10% of their primary structure [residues 35 and 34, respectively: Wasserman et al. (1984) and Galakatos et al. (1986)], our initial attempt at assigning sequence orientation to the proteolytic fragments entailed prior active site labeling. DadB holoenzyme was reduced with NaB^3H_4 and the subjected to controlled digest under standard conditions. SDS–PAGE analysis of the subtilisin digest followed by autoradiography revealed a single radioactive band corresponding to the 28-kDa fragment. Surprisingly, the reduced holoenzyme remained intact and kinetically resistant to digestion with α-chymotrypsin or trypsin when subjected to the same reaction conditions as the native holoenzyme (Figure 2).

The sequence locus of the proteolytic site was established as follows: Both pairs of fragments generated from α-chymotrypsin digests of DadB and Alr were isolated by preparative SDS–PAGE followed by electroelution and were then subjected to automated Edman degradation. In the case of the two 28-kDa fragments, their carboxyl termini were determined with carboxypeptidase A. The results are shown in Table I and clearly indicate that both DadB and Alr are clipped twice at vicinally oriented aryl amino acid residues (DadB, Tyr^{253} and Tyr^{257}; Alr, Tyr^{255} and Trp^{259}) to release in each case a tetrapeptide.

Residue-nonspecific subtilisin attacks native DadB at both the chymotryptic site Tyr^{257} and at neighboring Thr^{260}. On the basis of the preference of trypsin for Lys and Arg residues, the size of the tryptic digest fragments obtained, and the known overall protein sequence, it can be inferred that this protease also attacks the native enzymes twice (DadB, Arg^{250} and Arg^{264}; Alr, Lys^{248} and Arg^{263}), releasing a tetradeca- and a

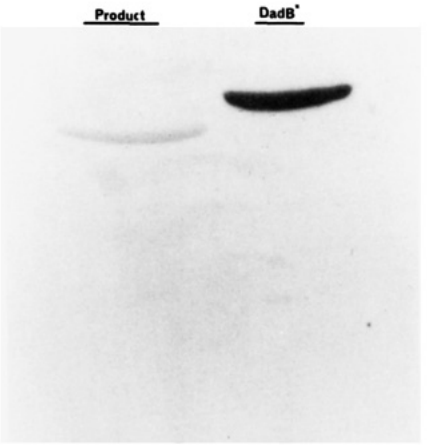

FIGURE 3: Inactivation of clipped two-domain DadB (3% active) with β-chloro-[^{14}C]-D-alanine followed by reduction with $NaBH_4$. Shown is the SDS–PAGE radioactivity profile of the protein product purified from this reaction mixture by gel filtration (Sephadex G-75, superfine): The single band at the product lane corresponds to the PLP-containing NH_2-terminal domain (M_r 28,000) of DadB; DadB* is the NaB^3H_4-reduced holoenzyme which was used as a radioactive size marker (M_r 39 000).

pentadecapeptide from DadB and Alr, respectively.

The high degree of proteolytic regioselectivity, the twin clipping, and the sequence of the peptides released are all very suggestive of a surface, structurally flexible hinge region connecting the NH_2- and CO_2H-terminal domains. For both isoenzymes, sequence-dependent hydrophilicity profiles (Eisenberg et al., 1982) predict that the experimentally determined hinge loci also constitute regions of maximum exposure to solvent.

Domain Association under Nondenaturing Conditions. The product of chymotryptic digest of DadB was treated with immobilized α_2-macroglobulin to remove the protease and then was passed through a gel filtration column. A single peak was observed, corresponding to a molecular weight of ca. 36 000. The pooled fractions were concentrated and then resolved by SDS–PAGE to cleanly afford two bands at M_r 28 000 and 11 000. These data taken together indicate that under nondenaturing conditions the two proteolytic domains remain associated. A similar result was obtained for clipped Alr. Following the purification procedure described above, both clipped two-domain enzymes retained the same level of catalytic activity noted at the end of the proteolysis reaction (3% of the native V_{max}). A variety of conditions employed to afford the dissociation of the two domains for both DadB and Alr (e.g., low pH, high [NaCl] in the eluent of the gel filtration column) failed to yield single polypeptide domains in native form.

Catalytic Activity Associated with the Clipped Two-Domain DadB. In order to ensure that the 3% activity is inherent to the proteolyzed DadB and not due to any residual intact holoenzyme, we studied the mode of its inactivation with the mechanism-based inactivating substrate β-chloro-[^{14}C]-D-alanine (Badet et al., 1984), which covalently derivatizes the PLP–lysine imine as a nonhydrolyzable ternary adduct of inactivator, coenzyme, and enzyme. When the clipped two-domain enzyme was quenched with the inactivator, complete loss of the inherent residual activity was observed. Following $NaBH_4$ reduction and separation of the fragments by SDS–PAGE, a single radioactive band corresponding to the active site containing NH_2-terminal domain (M_r 28 000) was detected. No trace of inactivated reduced holoenzyme was apparent (Figure 3). Calculation of the specific radioactivity of the inactivated, $NaBH_4$-reduced clipped two-domain DadB

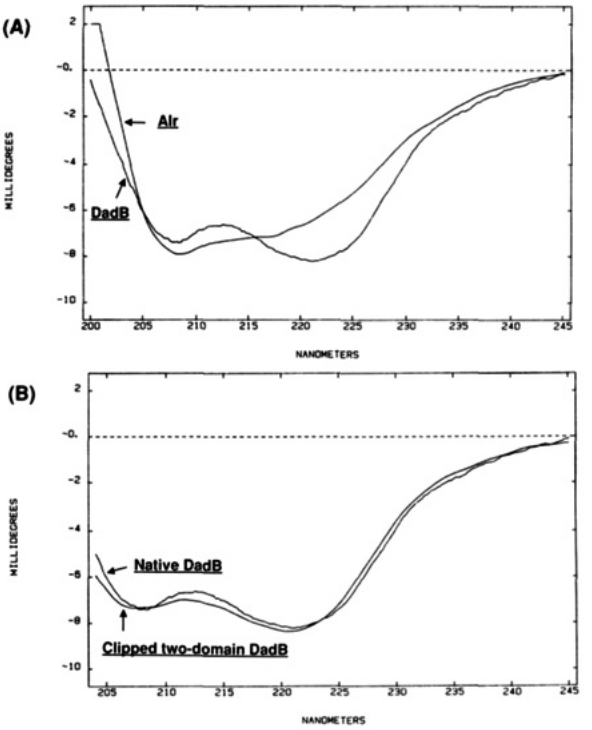

FIGURE 4: Circular dichroism spectra recorded in buffer C (Materials and Methods) at 1–4 mg/mL and then scaled computationally to 0.1 mg/mL. (A) Comparison between native DadB and native Alr alanine racemases. (B) Demonstration of conserved secondary structure between native DadB and its clipped two-domain derivative.

demonstrated the incorporation of 0.76 equiv of the label at the active site.

Circular Dichroism Studies. As shown in Figure 4, the two-domain and the native forms of the DadB alanine racemase appear to be indistinguishable by circular dichroism at the UV region. Although not preclusive of minor geometry perturbations in the protein, this result nevertheless indicates that the clipped enzyme retains its secondary structural elements intact. Comparison of the UV CD profiles of native Alr and DadB suggests similar α,β structure for the two proteins. By the curve-fitting method of Yang (Yang et al., 1986), DadB is predicted to possess the following fractions: α = 29% and β = 45%. For Alr, α = 31% and β = 48%.

DISCUSSION

The discovery in these laboratories of two isoenzymic alanine racemases from *S. typhimurium* (Wasserman et al., 1983) serving independently catabolic (DadB) and biosynthetic (Alr) functions and the subsequent extension of this observation by others to *E. coli* racemases (Wild et al., 1985) raise the question of differential regulation of L-alanine metabolism in bacteria.

For the racemases from *S. typhimurium* a genetic approach aimed at the elucidation of this issue at the transcription level has been undertaken (Daub, 1986). In parallel to this effort, DadB and Alr have been compared in terms of catalytic efficiency and mode of inactivation with mechanism-based inhibitors (Badet et al., 1984; Esaki & Walsh, 1986). It was found that the two isoenzymes differ in active site environments since DadB, although it converts L-alanine to D-alanine 60 times faster than Alr, when inactivated with β-haloalanines gives a value for the partition ratio of inactivation to turnover that is one-fifth of that determined for Alr (Esaki & Walsh, 1986). This observation is striking in view of our recent demonstration that the two isoenzymes possess identical active site decapeptides equally distanced from the NH_2 termini

Table II: Sequence Homology at the Interdomain Hinge Region of Alanine Racemases[a]

source	alanine racemase	M_r	no. of residues	hinge region[c]
S. typhimurium	DadB	1 × 39 000	356	-V-G-[Y253]-G-G-G-[Y257]-S-V-
	Alr	1 × 39 000	359	-V-G-[Y255]-G-G-T-[W259]-V-S-
B. stearothermophilus	Bsar	2 × 42 000	386	-V-S-[Y264]-G-A-T-[Y268]-T-A-
B. subtilis	Bdal	(43 000)[b]	389	-V-S-[Y266]-G-A-E-[Y270]-T-A-

[a] Overall sequence identities: DadB vs Alr, 43%; DadB vs Bdal, 39%; Alr vs Bdal, 35%. [b] Estimated from DNA-derived protein sequence; quaternary structure undetermined (Ferrari et al., 1985). [c] Boxes represent chymotryptic sites.

(DadB, PLP–Lys[35]; Alr, PLP–Lys[34]) and, overall, the two equivalently sized monomeric proteins exhibit 43% residue identity. However, despite the volume of available kinetic and primary sequence data, the molecular basis of catalytic and regulatory difference between DadB and Alr remains unclear.

Multifunctional enzymes are believed to be constructed by gene fusion (Kirschner & Bisswanger, 1976). In some cases, notably that of aspartokinase–homoserine dehydrogenase I and II, the gene-encoded activities reside in distinct compact assemblies of secondary structural elements defined as domains (Richardson, 1981) and can be separated experimentally by limited proteolysis (Fazel et al., 1983; Dautry-Varsat & Cohen, 1977). In some enzymes of single catalytic activity such as elastase, the structure is also subdivided in domains, this time defined as independently folding polypeptide units (Hogberg-Raibaud & Goldberg, 1977b; Ghelis et al., 1978). Such domain characterization through differential folding clearly suggests gene duplication, and although such domain assemblies in their present form appear to be syncatalytic, they may have evolved from precursors possessing separate enzymatic functions, such as catalysis and substrate binding.

The results presented in this paper indicate a two-domain structure for both alanine racemases from *S. typhimurium*, the domains being linked by a Gly-rich hinge that is recognized by specific and nonspecific proteases. The conservation of tertiary structure reported here and the published sequence homology (43% identity) between DadB and Alr (Galakatos et al., 1986), when considered in view of the distincly different chromosomal loci occupied by the respective racemase-encoding genes (*dadB*, minute 36; *alr*, minute 91), clearly suggest gene duplication (Cleveland et al., 1977). A striking observation is the conservation of the DadB and Alr hinge region sequence in the primary structure of alanine racemases from the gram-positive bacteria *B. subtilis* (Ferrari et al., 1985) and *B. stearothermophilus* (K. Tanizawa, personal communication) (Table II). The latter is a dimeric thermostable protein that as described under Results exhibits the same proteolytic pattern as the monomeric enzymes from *S. typhimurium*.

The cleavage of the interdomain bridge of DadB and Alr has been shown to result in a 30-fold drop in racemase activity without significant concurrent distortion of the active site geometry or disruption of secondary structure. The two domains remain associated in a functional form, although their through-the-backbone conformational mobility may have been compromised. Our result that the clipped protein incorporates the mechanism-based inactivator β-chloro-D-alanine at 76% of the available sites is suggestive that the active site of the great majority of the molecules in the population is catalytically functional. The activity and inactivation data taken together imply that at least 76% of the enzymatic molecules retain catalytic activity but their efficiency is reduced 30-fold to that of native racemase. The resistance of NaB^3H_4-reduced DadB holoenzyme toward α-chymotrypsin and trypsin demonstrates the profound effect of the change in oxidation states of the PLP-binding Lys[35] on hinge structure. One of two effects may be operating in this case: either the change in active site geometry is propagated through the protein to the interdomain bridge, or just simply the Gly-rich linker is spacially close to the cleft and its structure is directly affected by active site distortions.

The whole set of these results closely parallels the example of mitochondrial aspartate aminotransferase (AAT) from chicken heart (Sandmeier & Christen, 1980). Although tryptic digest of AAT released the 27-residue amino domain of the native enzyme and the PLP-binding lysine is at position 258 vs DadB Lys[35] and Alr Lys[34], in this equivalently sized dimeric protein (M_r 2 × 45 000) the remaining CO_2H-terminal domain (AAT residues 27–410) retained 30-fold less activity compared to the intact aminotransferase. From the known crystal structure of AAT it is clear that the proteolytic cleavage site consisting of the pentapeptide hinge -Arg[26]-Asp-Thr-Asn-Ser-Lys[31]- is located directly adjacent to the active site cleft (Ford et al., 1980). If the spacial proximity of the loop to the active site of AAT is conserved in DadB, then this will explain our result with the reduced holoenzyme. For AAT, it was also found that the proteolytic release of the NH_2-terminal 27 amino acid residue peptide did not affect the apparent K_M for the enzyme, which suggests that the substrate binding site was not distorted (Sandmeier & Christen, 1980). This result is consistent with the syncatalytic mode of action believed to operate with this PLP enzyme (Gehring & Christen, 1978). The determination of kinetic parameters associated with the clipped two-domain DadB racemase is in progress and will be reported elsewhere.

Structural domains have also been defined as independently folding units within a larger globular protein (Richardson, 1981). In the case of the PLP-requiring β_2-subunit of tryptophan synthetase from *E. coli* (M_r 2 × 45 000; 397 amino acid residues/subunit; ca. 21% protein sequence homology with DadB and Alr; Crawford et al., 1980), tryptic digestion generates two fragments (M_r 28 000 and 12 000) of which the larger bears the cofactor-binding Lys[87] (Hogberg-Raibaud & Goldberg, 1977a). Although inactive, the two tryptic fragments remain associated and in this form maintain the native tertiary structure and incorporate PLP at 80% of the available sites. Also, the sequence of the alanine racemase hinge is conserved within the Lys[272]-Lys[283] tryptophan synthetase interdomain linker (Tyr[279]-Phe-Gly-Met-Lys[283]), and a single cleavage at Arg[275] is sufficient to deactivate the native enzyme (Ahmed et al., 1986). More important however is the report that upon denaturation with 6 M urea and subsequent renaturation the two fragments fold independently to yield a structure that is identical with clipped tryptophan synthetase in the native form (Hogberg-Raibaud & Goldberg, 1977b); Zetina & Goldberg, 1980). The close analogy in proteolytic cleavage patterns between tryptophan synthetase and the alanine racemases from *S. typhimurium* suggests that the Gly-rich interdomain linker present in the latter enzymes may in fact connect two independently folding polypeptide fragments.

We plan to explore this possibility and also construct CO_2H-terminal domain deletion mutants (Jasin et al., 1983)

in order to establish possible domain–function relations that may allow the dissociation of the catalytic and substrate-binding functions of these enzymes. This future work should complement ongoing efforts by Professor Petsko's laboratory to obtain a crystal structure for the alanine racemase from *B. stearothermophilus* (Neidhart et al., 1987).

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