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REVIEW

Structure and Function of Alanine Racemase

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I. Introduction

Peptidoglycan is a major structural element that provides resistance to osmotic lysis in both Gram negative and positive bacteria, and contains D–amino acids which presumably provide stability against proteolytic degradation. Thus, in Gram negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*, peptidoglycan is assembled by a UDP–muramyl pentapeptide with four of the five amino acids in the pentapeptide containing D–amino acids (L–Ala–D–Glu–*meso*–diaminopimelate–D–Ala –D–Ala). This pentapeptide precursor is translocated into the membrane and cross–linked. While the ε–amino group of a *meso*–diaminopimelate residue is cross–linked directly to the penultimate D–Ala in a second pentapeptide strand in Gram negative bacteria, a pentaglycine bridge connects the strands in Gram positive bacteria. The new interstrand peptide bond is formed as the intrastrand D–Ala–D–Ala bond is broken. D–Alanine is a central molecule in the peptidoglycan assembly and cross–linking. Alanine racemase (EC 5.1.1.1) catalyzes the interconversion between L– and D–alanine and supplies D–alanine for the peptidoglycan synthesis.

Alanine racemase has been purified from *Pseudomonas putida* (2), *Bacillus subtilis* (3), *Salmonella typhimurium* (4, 5), *Streptococcus faecalis* (6), and *Bacillus stearothermophilus* (7). The enzyme requires pyridoxal 5′–phosphate (PLP) as a cofactor. PLP binds to the lysine residue of the enzyme protein and forms an aldimine Schiff base. The α–proton of the substrate moiety is then abstracted, and the PLP–substrate carbanion is generated. Stereorandom return of the proton yields racemic alanine. The mechanism of alanine racemase reaction have been studied thermodynamically by Faraci & Walsh (8).

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II. Genetical and evolutional aspects of alanine racemase

Walsh and his coworkers carried out the molecular cloning of alanine racemase genes (9). Two distinct *Salmonella* DNA clones that complement the D–alanine requirement of *E. coli* strain that is temperature sensitive for growth in the absence of D–alanine have been isolated (9). Both contain distinct alanine racemase genes; one mapped at minute 37 on the chromosome is termed the *dadB*, and the other mapped at minute 91 is termed the *alr* gene (10). The two distinct alanine racemase genes have been also demonstrated in *E. coli* (11). The DadB alanine racemase is inducibly formed and functions in the catabolism of L–alanine; D–alanine produced by racemization is deaminated to pyruvate by D–alanine dehydrogenase, whose gene

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1        10        20        30        40*       50        60
MSTKPFYRDTWAEIDLSAIKENVSNMKKHIGEHVHLMAVEKANAYGHGDAETAKAALDAG
  MNDFHRDTWAEVDLDAIYDNVENLRRLLPDDTHIMAVVKANAYGHGDVQVARTALERG
     MTRPIQASLDLQVMKQNLAIVRRAAP-EARVWSVVKANAYGHGIERV-WSALGAT
     MQAAT-VVINRRALRHNLQRLRELAP-ASKLVAVVKANAYGHGLLETART-LPDA

         70        80        90        100       110       120
ASCLAMAILDEAISLRKKGLKAPILVLGAVPPEYVAIAAEYDVTLTGYSVEWLQEA-ARH
-PPPAVAFLDEALALREKGIEAPILVLGASRPADAALAAQQRIALTVFRSDWLEEASALY
-DGFAMLNLEEAITLRERGWKGPILMLEGFFHAQDLEAYDTYRLTTCIHSNWQLKALQNA
-DAFGVARLEEALRLRAGGITQPILLLEGFFDAADLPTISAQCLHTAVHNQEQLAALEAV

         130       140       150       160       170       180
TKKGSLHFHLKVDTGMNRLGVKTEEEVQNVMAILDRNPRLKCKGVFTHFATADEKERGYF
SGPFPIHFHLKMDTGMGRLGVKDEEETKRIVALIERHPHFVLEGLYTHFATADEVNTDYF
RLNAPLDIYVKVNSGMNRLGFQPERAQTVWQQL-RAMRNVGEMTLMSHFAQAD--HPEGI
ELAEPVTVWMKLDTGMHRLGVRPEEAEAFYQRLTHCKNVRQPVNIVSHFARADEPECGAT

         190       200       210       220       230       240
LMQFERFKELIAPLPLKNLMVHCANSAAGLRLKKGFFNAVRFGIGMYGLRPSADMSDEIP
SYQYTRFLHMLEWLPSRPPLVHCANSAASLRFPDRTFNMVRFGIAMYGLAPSPGIKPLLP
GEAMRRIALATEGLQCAYSL---SNSAATLWHPQAHYDWVRPGIILYGASPSGQWRDIAD
EHQLDIF---NAFCQGKPGQRSIAASGGILLWPQSHFDWARPGIILYGVSPLEHKPWGPD

         250       260       270       280       290       300
FQLRPAFTLHSTLSHVKLIRKGESVSYGAEYTAEKDTWIGTVPVGYADGWLR-KLKGTDI
YPLKEAFSLHSRLVHVKKLQPGEKVSYGATYTAQTEEWIGTIPIGYADG-VR-RLQHFHV
TGLKPVMTLSSEIIGVQTLSAGERVGYGGGYSVTQEQRIGIVAAGYADGYPRHAPTGTPV
FGFQPVMSLTSSLIAVRDHKAGEPVGYGGTWVSERDTRLGVVAMGYGDGYPRAAPSGTPV

         310       320       330       340       350       360
LVKGKRLKIAGRICMDQFMVELDQEYPP--GTKVTLIGRQGDEYISMDEIAGRLETINYE
LVDGQKAPIVGRICMDQCMIRLPGPLPV--GTKVTLIGRQGDEVISIDDVARHLETINYE
LVDGIRTRTVGTVSMDMLAVDLTPCPQAGIGTPVELWG---KE-IKVDDVASAAGTLGYE
LVNGREVPIVGRVAMDMICVDLGPNAQDNAGDPVVLWG----EGLPVERIAEMTKVSAYE

         370       380       390
VACTISSRVPRMFLENGSIMEVRNPLLQVNISN ---------B. subtilis
VPCTISYRVPRIFFRHKRIMEVRNAIGRGESSA ---------B. stearothermophilus
LLCAVAPRVPFVTT--------------------------------S. typhimurium dadB
LITRLTSRVAMKYID ------------------------------S. typhimurium alr
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Fig. 1

(*dad*A) is located adjacent to *dad*B and repressed together with *dadB* and *dad*R (9). The other alanine racemase, Alr enzyme, is constitutively synthesized and functions in anabolic assembly of peptidoglycan (10). Mutation in either one of the alanine racemase genes does not result in D–alanine auxotrophy in *S. typhimurium*, but mutants lacking both genes require exogenous D–alanine for growth (10).

We made a comparison among the complete amino acid sequences of alanine racemase from four different sources (*B. stearothermophilus* (12) ; *B. subtilis* (11) ; *S. typhimurium*, DadB ; *S. typhimurium*, Alr) in order to find out the homologous regions in their primary structures (Fig. 1). Despite the fact that *Bacillus* and *Salmonella* belong to Gram–positive and Gram–negative bacteria, respectively, and that the *dad*B and *alr* genes map at two distinctly different regions of the *S. typhimurium* chromosome, the four racemase sequences display considerable homology : 74 residues match in the sequences of the four enzyme and 53 residues match in the sequences of three (about 33 % homology on average for the sequences of four enzymes). The sequence homologies between the two of four racemases were calculated as 54 % (*B. stearothermophilus* : *B. subtilis*) ; 43 % (DadB : Alr) ; 35 % (*B. stearothermophilus* : DadB) ; 34 % (*B. subtilis* : DadB) ; 31 % (*B. stearothermophilus* : Alr) ; and 30 % (*B. subtilis* : Alr). The high homology between DadB and Alr strongly suggests that they evolved from a common ancestor by gene duplication. An octapeptide containing the active–site lysyl residue that binds PLP occurs in all the four enzymes. Low–specificity amino acid racemase of *P. putida* contains the same sequence, and also probably evolved from the common progenitor.

III. Structure and function of thermostable alanine racemase of *Bacillus stearothermophilus*

1 . Subunit dissociation and unfolding

The *B. stearothermophilus* alanine racemase is quite stable upon heat treatment at 70 ℃ for 80 min in 10 mM potassium phosphate buffer (pH 7.2). This thermostability of the enzyme enable us to subject the enzyme to high–resolution X–ray analysis, which is now in progress (13).

Alanine racemase of *B. stearothermophilus* consists of two identical subunits, whereas both DadB and Alr enzymes of *Salmonella typhimurium* and also the *Streptococcus faecalis* enzyme occur as a monomer. Toyama *et al.* examined whether the monomeric form of the *B. stearothermophilus* enzyme is catalytically active or not. They studied the guanidine HCl–induced subunit dissociation and unfolding of the enzyme by means of circular dichroism (CD) analysis, fluorescence and absorption spectroscopies, and gel filtration (14). The overall process was found to be reversible : more than 75 % of the original activity was recovered in a decrease in the denaturant concentration. The enzyme was unfolded by guanidine HCl treatment through two detectable phases : Phases 1 and 2 were observed by fluorescence spectroscopy and gel filtration, and Phase 2 by CD measurement. They concluded that the two phases are derived from the following transitions : Phase 1, dissociation of the dimer to monomers ; Phase 2, unfolding of the monomer (14).

The monomeric form of the protein was found to be catalytically inactive (14). The absorption spectrum of the monomeric form indicated that the carbonyl group of PLP is not bound in an internal Schiff base linkage. The reactivity of the carbonyl group of PLP is usually elevated by formation of a Schiff base with the ε–amino group of a lysyl residue at the active site of PLP enzymes (15). The mutant enzymes of aspartate aminotransferase (16) in which the PLP–binding lysyl residue is replaced by arginine do not react with PLP to form a Schiff base, but they show a slight, but distinct activity. Toyama *et al.* suggested that the inactivity of the monomeric form is due to a local and minor structural change which simultaneously occurs with the subunit dissociation (14).

The thermostable alanine racemase undergoes a major structural change when guanidine HCl concentrations are increased to 1.8 M. The unfolded monomer aggregates with each other to form a species with a high molecular weight. Isolated monomers of β_2–subunit of tryptophan synthase (17) and aspartate aminotransferase of *E. coli* (18) also aggregate.

The subunit dissociation is usually accompanied by destruction of the secondary structures when thermolabile proteins are used. The thermostable alanine racemase is a feasible material to study the mechanism of subunit dissociation and protein unfolding.

2. Limited proteolysis

Galakatos and Walsh showed that the native DadB and Alr racemases are digested at homologous positions by α–chymotrypsin, trypsin, and subtilisin to produce two non–overlapping polypeptides of *Mr* 28,000 and 11,000 (19). Both enzymes are composed of two domains, which are linked by a hinge. The two domains are associated with each other after the cleavage at the interdomain bridge under denaturing conditions. Both clipped enzymes retain about 3 % of the original activity ; the active–site geometry and secondary structure are not distorted by the proteolysis. The hinge region is conserved also in the primary structure of alanine racemases from *Bacillus subtilis* and *B. stearothermophilus* (19).

Toyama *et al.* showed that the *B. stearothermophilus* enzyme also is cleaved into two major fragments with *Mr* 14,000 (fragment F–1) and 29,000 (F–2) with subtilisin (20). They isolated F–1 and F–2 fragments by reversed phase HPLC, and showed that the enzyme is cleaved by proteolysis around ^{264}Tyr to produce F–1 and F–2 based on the amino acid compositions of the isolated fragments. They showed also that approximately 85 % of the native protein disappeared after incubation for 72 h, and that about 50 % of the original activity remained (20). This indicates that about 15 % of the native enzyme was unchanged, and that the activity corresponding to 35 % of the original activity was derived from the peptide fragments formed. The nicked forms of both DadB and Alr enzymes were only 3 % as active as the corresponding native forms (19). Thus, the peptide fragments of the *B. stearothermophilus* enzyme interact with each other more strongly to form a considerably active structure than that of the thermolabile enzyme. Toyama *et al.* constructed and expressed a mutant gene which tandemly encodes the two peptides correspond-

ing to F–2 and F–1 (21).

3. Construction and expression of fragmentary enzyme

To examine whether the two polypeptide fragments generated by limited proteolysis correspond to structural domains, Toyama *et al.* attempted to prepare the two polypeptides by means of genetic engineering (21). They constructed a mutant alanine racemase gene of *B. stearothermophilus*, which contains a new set of termination and initiation codons in the position of the gene corresponding to the putative hinge region of DadB and Alr racemases. The gene was found to be expressed to form an active alanine racemase composed of two dissimilar polypeptides (21). The mutant enzyme named fragmentary alanine racemase, was active in both directions of the racemization of alanine: the maximum velocity (Vmax) was about half of that of the wild–type enzyme; the Km value was about twice. Absorption and CD spectra of the fragmentary enzyme were also similar to those of the wild–type enzyme. Since the fragmentary enzyme was considerably active, Toyama *et al.* suggested that its overall conformation is identical with that of the wild–type enzyme (21). Conformational differences, if any, should be confined to a local region at or in the vicinity of the active site, judging from the slight difference in spectral properties of the cofactor bound to the active site. These findings led to a conclusion that the two polypeptide fragments correspond to structural folding units (domains) in the parental polypeptide chain of alanine racemase as shown in Fig. 2.

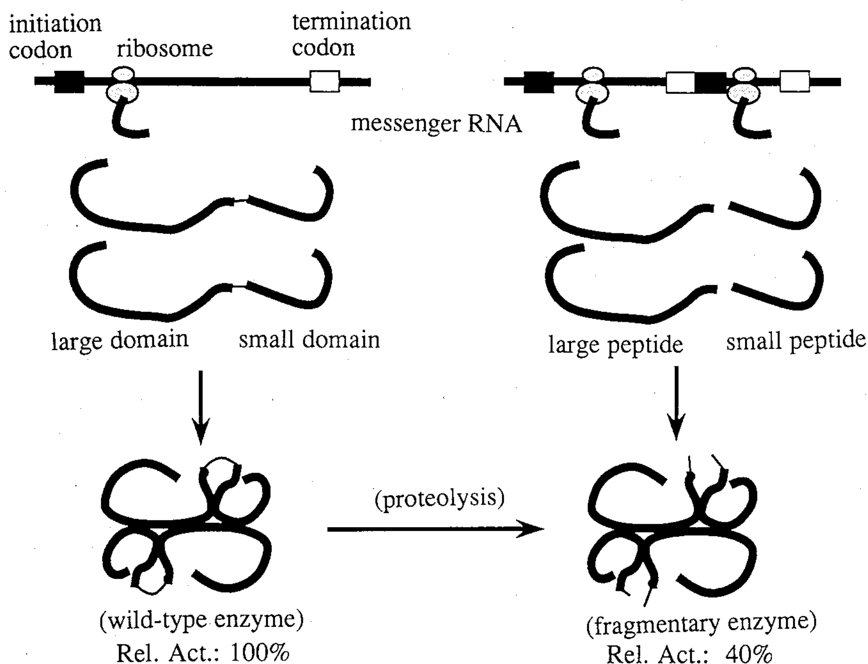

Fig. 2

Although the overall conformation of the fragmentary enzyme appears to be identical with that of the wild-type enzyme, it has only 40 % of the activity of the native enzyme. The decreased activity of the fragmentary enzyme was accounted kinetically to a combined consequence of both the increase in Km values for substrates and the decrease in the Vmax values as described above (21). Galakatos and Walsh (22) showed by site-directed mutagenesis studies that the hinge region in the DadB alanine racemase may play a role as a pivot for movement of the two domains during catalysis and may be located close to the active site, interacting with the bound PLP. Toyama *et al.* suggested that the decreased activity of the fragmentary enzyme relative to that of the native enzyme is due to either the presence of several extra residues in the hinge region or the discontinuity of the hinge sequence itself (21). In accordance with this view, one of the DadB mutant enzymes in which a fourth Gly was inserted into the intraloop tetrapeptide, showed a 5-fold drop in Vmax/Km (22).

The relative activity of the fragmentary enzyme to the wild-type enzyme was much higher than that of the clipped to the native DadB enzyme. This was explained by the assumption that the thermostable enzyme has more extensive hydrophobic interdomain interactions than the DadB enzyme with less thermostability (12). The importance of hydrophobic interdomain interactions for catalytic activities was pointed out by studies on lactate dehydrogenase (23, 24).

Toyama *et al.* attempted to produce a single polypeptide corresponding to each domain separately in *E. coli* (21). However, no protein reactive with the antibody against the wild-type alanine racemase was produced. They suggested that the fragments expressed in separate host cells are broken down proteolytically in the cells after the translation (21). The production of a similar N-terminal fragment from the DadB enzyme was also unsuccessful (22). The two peptide fragments probably fold into an active structure only when they are co-translated. To examine whether the folding process of each fragment into the domain structure is cooperative with each other during translation, Toyama *et al.* attempted to establish the in vitro conditions for reversible resolution of the fragmentary enzyme into two polypeptides and their reconstitution into the active enzyme (25).

4. Unfolding and reconstitution of fragmentary enzyme

Toyama *et al.* showed that the fragmentary alanine racemase of *B. stearothermophilus* is unfolded by treatment with guanidine HCl through two detectable phases in the same manner as the wild-type enzyme: Phase 1, dissociation into two $\alpha\beta$-forms; Phase 2, dissociation into two fragments and unfolding of each fragment. However, they found no distinct intermediate processes between the subunit dissociation and the unfolding of the large and the small fragments.

They isolated the two polypeptides from the fragmentary alanine racemase in the presence of 4 M guanidine HCl, and attempted to reconstitute and reactivate the enzyme. The polypeptides were refolded spontaneously into an active form upon removal of guanidine HCl. The spectra obtained by summation of the CD spectra of the isolated subunits was close to that of the native fragmentary enzyme. The

lysine residue to which PLP is bound in the wild-type enzyme occurs in the large subunit of the fragmentary enzyme. However, neither bound PLP nor activity was found in the large subunit. Both fragments need to be folded together in order to form an active structure comparable with the native fragmentary enzyme. The fine protein conformation necessary for the catalytic activity is probably formed only through an interchain association of both fragments.

IV. References

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