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# Mutational Scanning of a Hairpin Loop in the Tryptophan Synthase β-Subunit Implicated in Allostery and Substrate Channeling

**Philippe Rondard and Hugues Bedouelle***

Unité de Biochimie Cellulaire, CNRS-URA2185,
Institut Pasteur, 28 rue Docteur Roux,
F-75724 Paris Cedex 15, France

* Corresponding author

**The tryptophan synthases from *Escherichia coli* and *Salmonella typhimurium* are tetrameric enzymes, with an elongated TrpA.TrpB.TrpB.TrpA structure. Structural studies have identified residues 277–283 of TrpB as a potentially important region for the allosteric communication between the TrpA and TrpB subunits and for the transport of indole between their active sites through a hydrophobic tunnel. To explore the functional role of this region, we analyzed the effects of 19 single and double mutations in TrpB on the tryptophan synthase (TSase) and serine deaminase (SDase) activities of the $TrpB_2$ dimer, either in the presence or in the absence of the TrpA subunit. The mutations of residues 273–283 could be divided into 4 classes. Mutations I278A, F280G and M282A decreased the SDase and TSase activities of $TrpB_2$ to similar extents. F280A decreased the SDase activity of $TrpB_2$ more than its TSase activity, whereas the reverse was true for Y279L. F280A decreased the activation factor of $TrpB_2$ by TrpA, whereas F280G increased it. The reaction steps and intramolecular contacts that could be affected by the mutations are described. The sequence 278-IYFGM-282, which is present in *E. coli* and *S. typhimurium*, is only found in 5 out of 42 organisms, whereas the sequence VLHGX is found in 21 organisms. Our results identified several mutations that could be used as structural probes to analyze precisely the roles of residues 278–282 and their evolution.**

*Key words:* Enzyme activation / *Escherichia coli* / Indole / *Salmonella typhimurium* / Serine deaminase / Tunnel.

## Introduction

Tryptophan synthase catalyzes the last two steps in the biosynthesis of L-tryptophan (for reviews see Yanofsky and Crawford, 1972; Miles, 1995; Pan *et al.,* 1997; Anderson, 1999). The two subunits, TrpA and TrpB, form a bifunctional complex, which has the composition $TrpA_2.TrpB_2$. The enzymes from *Salmonella typhimurium* and *Escherichia coli* are very similar because their amino acid sequences are 86% identical for TrpA and 96% identical for TrpB (Nichols and Yanofsky, 1979; Crawford *et al.,* 1980). The TrpA subunit catalyzes the conversion of indole-3-glycerol phosphate (IGP) to glyceraldehyde 3-phosphate (G3P) and indole. The TrpB subunit catalyzes the condensation of indole and L-serine into L-tryptophan. Although the TrpA monomer and the $TrpB_2$ dimer, in their unassociated states, are relatively inefficient catalysts, their catalytic efficiencies and affinities for the substrates increase by one or two orders of magnitude when the proteins are in the form of a complex. The $TrpB_2$ dimer, unassociated with TrpA, also catalyzes the conversion of L-Ser to pyruvate, but this reaction is not catalytically significant in the $TrpA_2.TrpB_2$ complex (Yanofsky and Crawford, 1972):

IGP ↔ G3P + indole (α-reaction)
indole + L-Ser ↔ L-Trp + $H_2O$ (β-reaction)
L-Ser → pyruvate + $NH_3$ + $H_2O$ (serine deaminase reaction)

The β-reaction and the serine deaminase reaction depend on the coenzyme pyridoxal 5′-phosphate (PLP), which forms a Schiff base with the ε-amino group of residue βLys87 in the free TrpB subunit (also called internal aldimine). The two reactions proceed through two intermediate Schiff bases: between PLP and L-Ser (external aldimine of L-Ser) and between PLP and amino-acrylate. The two reaction pathways then diverge, towards L-Trp in the presence of indole and towards pyruvate in its absence (Miles, 1995). In the $TrpA_2.TrpB_2$ complex, the presence of the intermediate amino-acrylate at the active site of the TrpB subunit (β-site) activates the α-reaction. Once an indole molecule is formed at the active site of the TrpA subunit (α-site), it is transferred rapidly to the β-site where it reacts with the amino-acrylate to form L-Trp. The allosteric communication between the α- and β-sites keeps the α- and β-reactions in phase, such that the intermediate indole does not accumulate (Pan *et al.,* 1997; Anderson, 1999; Miles *et al.,* 1999). The crystal structure of the Trp-synthase from *S. typhimurium* has revealed an extended arrangement of the subunits, TrpA.TrpB.TrpB.TrpA, and the existence of a 25 Å long hydrophobic tunnel, connecting the α- and β-sites. It has been proposed that indole, the product of the α-reaction, diffuses to the β-site through this tunnel. In contrast, L-Ser enters and L-Trp exits the β-site *via* a direct opening to the solvent (Hyde *et al.,* 1988).

Several structural studies have pointed out residues β277 to β283 of TrpB as an important region for the dynamics of Trp-synthase and the passage of indole through

**Table 1** Structures of the Trp-Synthase Variants from *S. typhimurium* Used in This Study.

| PDB file | Mutation | Cation | Ligands | Resolution (Å) | Reference |
|---|---|---|---|---|---|
| 1TTQ | wt | $K^+$ | PLP | 2.0 | Rhee *et al.*, 1996 |
| 1A5A | αD60N | $K^+$ | PLP | 1.9 | Rhee *et al.*, 1998a |
| 1BEU | αD60N | $K^+$ | PLP, L-Ser, IPP | 1.9 | Rhee *et al.*, 1998a |
| 1A5B | αD60N | $K^+$ | PLP, IGP | 2.0 | Rhee *et al.*, 1998a, b |
| 1TTP | wt | $Cs^+$ | PLP | 2.3 | Rhee *et al.*, 1996 |
| 1BKS | wt | $Na^+$ | PLP | 2.2 | Hyde *et al.*, 1998 |
| 1UBS | βK87T | $Na^+$ | PLP, L-Ser | 1.9 | Rhee *et al.*, 1997 |
| 2TYS | βK87T | $Na^+$ | PLP, L-Trp | 1.9 | Rhee *et al.*, 1997 |
| 2TSY | βK87T | $Na^+$ | PLP, L-Ser, GP | 2.5 | Rhee *et al.*, 1997 |
| 2TRS | βK87T | $Na^+$ | PLP, L-Ser, IPP | 2.04 | Rhee *et al.*, 1997 |
| 2WSY | wt | $Na^+$ | PLP | 2.3 | Schneider *et al.*, 1998 |
| 1A50 | wt | Na+ | PLP, F-IPP | 2.3 | Schneider *et al.*, 1998 |
| 1A5S | wt | Na+ | PLP, A-A, F-IPP | 2.3 | Schneider *et al.*, 1998 |

Abbreviations: PLP, L-Ser, L-Trp and A-A, Schiff bases between pyridoxal 5′-phosphate and βLys87, L-Ser, L-Trp or amino-acrylate, respectively; GP, DL-α-glycerol 3-phosphate; IGP, indole-3-glycerol phosphate; IPP, indole-3-propanol phosphate; F-IPP, 5-fluoroindole propanol phosphate; PDB, Protein Data Bank.

its hydrophobic tunnel. The crystal structures of the wild-type Trp-synthase, $TrpA_2.TrpB_2$(wt), in its free form, in complex with 5-fluoroindole propanol phosphate (F-IPP, an inhibitor of the α-site), and in complex with both F-IPP and amino-acrylate have been compared (Table 1). This comparison has revealed a pathway of allosteric communication between the α- and β-sites. A central element of this pathway is constituted by a rigid but mobile domain, called COMM, which comprises residues βGly102 to βGly189, *i.e.* the structural elements S3-L3-S4-H5-S5-H6-S6 of TrpB (H, α-helix; L, loop; S, β-strand). The chain of interactions is the following: α-site ↔ loops αL2 and αL6 of TrpA ↔ helix βH6 of COMM ↔ loop βL3 of COMM ↔ β-site (Schneider *et al.*, 1998). The binding of F-IPP to the α-site induces the formation of new H-bonds between residue βTyr279 and two residues of the COMM domain, *i.e.* βLys167 and βAsn171 of helix βH6. These H-bonds establish a link between the α-site and the pair of aromatic residues βTyr279-βPhe280, which are anchored in the wall of the indole tunnel (Schneider *et al.*, 1998).

A comparison of the crystal structures of $TrpA_2.TrpB_2$(wt) in the presence of $Na^+$, $K^+$ or $Cs^+$ ions has shown that the side chains of residues βTyr279 and βPhe280 move from a position that partially blocks the tunnel in the structure with $Na^+$, to a position that lines the surface of the tunnel in the structures with $K^+$ or $Cs^+$. As the residues of the cation binding site (βPhe306, βSer308, βVal231 and βGly232) either are closely linked to residue βAsp305 of the β-site or are involved in the binding of PLP, this structural comparison has suggested the existence of an allosteric link between the β-site and residues βTyr279-βPhe280 *via* the cation binding site (Table 1; Rhee *et al.*, 1996). The belonging of residue βAsp305 to the β-site and its involvement in the allosteric communication between the TrpA and TrpB subunits have been shown by structural and mutagenesis studies (Ahmed *et al.*, 1991; Rhee *et al.*, 1996, 1997; Schneider *et al.*, 1998). Solid-state NMR experiments, in which the TrpB subunits of Trp-synthase contained both [$^{19}$F]Phe and [$^{13}$C]Tyr, have shown that the conformation of βPhe280 changes when L-Ser binds to the β-subunit, but that βTyr279 and βPhe280 do not move relative to each other (McDowell *et al.*, 1996).

The dominant modes of movement that operate during the allosteric transition of $TrpA_2.TrpB_2$ have also been analyzed with a Gaussian network model. This dynamic study has shown the existence of a cascade of coordinated movements that all lead from the substrate at the β-site to the core of the COMM domain (residues β130-β145 and β155-β170). The movements of this core are strongly coordinated with those of residues β174-β179, which are located in helix βH6, at the junction between the two halves of the hydrophobic tunnel. This cascade of coordinated movements then rejoins residues α54-α60 of loop αL2 and the α-site through several different pathways: directly, or through residues β18-β44 of helices βH1 and βH2, or through residues α179-α183 of loop αL6, or through

residues β277-β283 of loop βL8 (Bahar and Jernigan, 1999).

A few mutations involving residues β273-β283 have been generated and studied in Trp-synthase. These mutations either replace a hydrophobic residue by polar ones as for βF280S and βF280C in the *S. typhimurium* enzyme (Ruvinov *et al.,* 1995), or add a bulky side chain as for βG281R in the *E. coli* enzyme (Zhao and Somerville, 1992, 1993). In a previous study we have analyzed the interaction between monoclonal antibody mAb164, directed against the $TrpB_2$ subunit of *E. coli,* and its epitope, located between residues β273 and β283 of TrpB, by a mutational approach. We mutated each of the residues β273-β283 into shorter residues, to proceed by simple deletion of side-chain groups; we also mutated some residues into Pro to modify the conformation of the polypeptide segment more globally, and then we measured the effects of the 16 single and 3 double mutations thus constructed on the free energy of interaction between mAb164 and $TrpB_2$ (Rondard and Bedouelle, 1998).

Given the possible involvement of residues β277-β283 in the dynamics of Trp synthase and the channeling of indole between the α- and β-sites, we thought it would be interesting to determine the effects of our previously constructed mutations on the activities of the $TrpB_2$ subunit and on their activation or repression by TrpA. In the present study, we measured the serine deaminase and Trp-synthase activities of each mutant $TrpB_2$, either in the absence or in the presence of the TrpA subunit. The results indicated that several mutations of residues β278-β282 could constitute valuable structural probes to deepen our understanding of the allosteric communication between the α-site, the β-site and the indole tunnel, and the way in which this communication was conserved or modified during evolution.

## Results and Discussion

### Classification of the Mutant $TrpB_2$ Dimers

We measured two specific activities for each mutant $TrpB_2$ dimer, its serine deaminase (SDase) activity and its tryptophan synthase (TSase) activity in the β-reaction. These two activities were measured either in the absence of TrpA (and then noted $SDase^-$ and $TSase^-$), or in the presence of an excess of TrpA (and then noted $SDase^+$ and $TSase^+$) (Table 2). We considered that a mutation decreased the SDase or TSase specific activity significantly if

$$SDase(mut)/SDase(wt) < 0.3, \quad (1)$$

$$TSase(mut)/TSase(wt) < 0.3, \quad (2)$$

(wt, wild type; mut, mutant). We considered that a mutation decreased the SDase activity significantly more than the TSase activity if

$$SDase(mut)/SDase(wt) < 0.5*TSase(mut)/TSase(wt). \quad (3)$$

Finally, we considered that a mutation decreased the activation of the β-reaction by TrpA significantly if

**Table 2.** Effects of Mutations of Residues β273-β283 on the Activities of $TrpB_2$ and Their Classification.

| Mutation | Class | $SDase^-$ U/mg (%) | $TSase^-$ U/mg (%) | $TSase^+$ U/mg | $TSase^+/^-$ fold |
|---|---|---|---|---|---|
| WT | | 47 (100) | 54 (100) | 1524 | 28 |
| H273A | IV | 121 (257) | 111 (206) | 1627 | 15 |
| R275A | IV | 50 (106) | 34 (63) | 1551 | 46 |
| V276A | IV | 38 (81) | 29 (54) | 1607 | 55 |
| I278V | IV | 51 (109) | 50 (93) | 1750 | 35 |
| I278A | I | 9 (19) | 16 (30) | 1553 | 97 |
| Y279F | IV | 27 (57) | 36 (67) | 1856 | 52 |
| Y279L | III | 48 (102) | 28 (52) | 2149 | 77 |
| Y279A | IV | 23 (49) | 27 (50) | 1868 | 69 |
| Y279P | II | 3 (6) | 29 (54) | 207 | 7 |
| F280A | II | 9 (19) | 56 (104) | 668 | 12 |
| F280G | I | 7 (15) | 15 (28) | 1236 | 82 |
| F280P | I | 3 (6) | 3 (6) | 480 | 160 |
| G281A | IV | 19 (40) | 29 (54) | 1485 | 51 |
| M282A | I | 14 (30) | 21 (39) | 1574 | 75 |
| M282P | III | 4 (9) | 0 (0) | 54 | ∞ |
| K283A | IV | 31 (66) | 32 (59) | 1557 | 49 |
| V276A,K283A | II | 11 (23) | 28 (52) | 1360 | 49 |
| I278A,K283A | I | 4 (9) | 5 (9) | 461 | 92 |
| I278V,K283A | IV | 21 (45) | 35 (65) | 1279 | 37 |

Abbreviations: $SDase^-$ and $TSase^-$, specific activities of $TrpB_2$ for the serine deaminase reaction and the β-reaction, respectively, in the absence of TrpA. The percentages relative to the wild-type activities are indicated between brackets. $SDase^+$ and $TSase^+$, activities in the presence of TrpA. The $SDase^+$ activities comprised between 3 and 8 U/mg for all the $TrpB_2$ species and are not shown. $TSase^+/^-$, ratio of the $TSase^+$ and $TSase^-$ activities (*i. e.* activation factor of $TrpB_2$ by TrpA for the β-reaction). ∞, large but undetermined value.

$TSase^+(mut)/TSase^-(mut) < 0.5*TSase^+(wt)/TSase^-(wt)$. (4)

Note that the terms (3) and (4) are independent of the protein concentrations. The mutations could be distributed into four classes by using these criteria (Table 2).

### Mutation Classes and Reaction Steps

The mutations of class I decreased the $SDase^-$ and $TSase^-$ activities significantly and by similar factors. They could affect reaction steps that are common to both activities, for example those leading to the amino-acrylate, since the pathways of the SDase and β-reactions diverge after this common intermediate. The mutations of class II decreased the $SDase^-$ activity more than the $TSase^-$ activity. They could affect reaction steps that are specific to the $SDase^-$ activity, *i. e.* those going from amino-acrylate to pyruvate. Alternatively, they could affect the same steps as the mutations of class I but simultaneously speed up the transport of indole from the external medium down to the β-site, through the portion of its tunnel which is included in $TrpB_2$. The belonging of mutation βF280A to class II is compatible with this second mechanism since residue βPhe280 is anchored in the wall of the indole tunnel (Rhee *et al.*, 1996). The mutations of class III decreased the $TSase^-$ activity more than the $SDase^-$ activity. They could affect steps that are specific to the $TSase^-$ activity, *i. e.* those going from amino-acrylate to tryptophan. Alternatively, they could slow down the transport of indole through its tunnel. The belonging of the conservative change βY279L to class III is compatible with this second mechanism since residue βTyr279 is anchored in the wall of the indole tunnel, in proximity of its opening at the surface of $TrpB_2$ (Rhee *et al.*, 1996). The mutations of class IV did not affect the $SDase^-$ and $TSase^-$ activities significantly.

The mutations of classes I and II showed the existence of a communication between residues β278-β282 and the β-site. Only three mutations, βH273A, βY279P and βF280A, decreased the factor by which TrpA activated the β-reaction. In the case of βH273A, the activation factor was low because the $TSase^-$ activity was higher for the mutant than for the wild type. The two other mutations, βY279P and βF280A, belonged to class II: they strongly decreased the $SDase^-$ activity and left the $TSase^-$ activity unchanged.

The channeling of indole from the α-site to the β-site through the non-polar tunnel of the $TrpA_2.TrpB_2$ complex is very fast and its rate (> 1000 $s^{-1}$) is not limiting for the α+β reaction. However, the binding of indole from the external medium to the α-site is slow and can be rate limiting for the β-reaction, with a second order rate constant equal to 2 $\mu M^{-1} \cdot s^{-1}$ (Anderson *et al.*, 1991). The rate constant for the entrance of indole from the external medium into the portion of its tunnel which is included within $TrpB_2$ is unknown and could also be rate limiting. In this hypothesis, mutations of residues βTyr279 and βPhe280 could affect differently the SDase and TSase activities through the transport of indole.

### Residue Variations between Organisms

Figure 1 shows an alignment of residues β273-β283 of the TrpB protein from *E. coli* with the homologous residues in the TrpB proteins from 41 other organisms. Residues βGly274, βGly277 and βGly281 are totally conserved. Compatibly, we found that the mutant $TrpB_2$(βG281A) dimer had lower $SDase^-$ activity (40%) and $TSase^-$ activity (54%) than $TrpB_2$(wt) (Table 2). The mutant $TrpB_2$(βG281R) has been studied in details (Zhao and Somerville, 1992, 1993). The mutation βG281R blocks the $TSase^-$ activity of $TrpB_2$ after the formation of an external aldimine with L-Ser. This blocking can be compensated by the presence of the TrpA subunit or of ammonium ions. In the presence of such ions, mutation βG281R increases the kinetic parameter $K_m$(L-Ser) of the β-reaction from 1.46 mM for $TrpB_2$(wt) to 25.4 mM for $TrpB_2$(βG281R), and $K_m$(indole) from 0.023 mM to 0.21 mM, but it has little effect on $V_{max}$. The limited effects that we observed for mutation βG281A could have two reasons. The mutation βG281R adds more atoms to the side chain than βG281A and could perturb the structure of the enzyme more. We used high concentrations of the substrates in our activity assays, 50 mM L-Ser and 0.5 mM indole, that could mask the consequences of an increase in $K_m$ on the reaction rates.

The residues in positions β278 (27 organisms with Val, 13 Ile, 1 Tyr and 1 Ala), β279 (28 Leu, 6 Phe, 5 Tyr and 3 Ile) and β280 (36 His and 6 Phe) are strongly conserved. The weak effect of mutation βI278V on the activities of $TrpB_2$, the moderate effects of βY279F and βY279L, and the significant effects of βI278A, βF280A and βF280G were compatible with the patterns of conservation for the three corresponding residues. The residues in positions β273 (8 possible side chains), β275 (9), β276 (8), β282 (7) and β283 (7) are the least conserved. The low effects of the mutations at these positions on the activities of $TrpB_2$ were compatible with the lack of conservation for the corresponding residues, except for βMet282.

The consensus sequence for residues β278-β282 is 278-VLHGX-282, where X can be any of 7 residues. This sequence is found 21 times among the 42 organisms analyzed. βPhe280 is found in 6 organisms either within the sequence 278-IYFGM-282 (5 cases) or within 278-IFFGM-282 (1 case). Remarkably, β282 is Met each time β280 is Phe, and β280 is Phe each time β279 is Tyr. The evolutionary intermediates VLHGM, ILHGM, IFHGM, IFFGM and IYFGM all exist.

### Accessibility to the Solvent

To explain the variability of residues β273-β283 between organisms and the effects of their mutations on the activities of $TrpB_2$, we analyzed their structural environment in the Trp-synthase from *S. typhimurium*. We first calculated the surface area that is accessible to the solvent (ASA), for the side chains of residues β273-β283 in the context of the $TrpA_2.TrpB_2$(wt, $K^+$) complex or of the isolated $TrpB_2$(wt, $K^+$) dimer (Figure 1). We used the structure of the $TrpB_2$ dimer in the $TrpA_2.TrpB_2$(wt, $K^+$) complex as a model for the

| | | |
|---|---|---|
| MAIZE1 | K G Q V G V L H G S M | 280 |
| MAIZE2 | K G Q V G V L H G S M | 334 |
| ARATH1 | K G D V G V L H G A M | 361 |
| ARATH2 | K G D V G V L H G A M | 366 |
| CAMAC | K G E V G V L H G A M | 357 |
| SYNY3 | M G K P G V L H G A M | 301 |
| CHLRE | M G T P G V L H G S Y | 335 |
| PSEAE | G G V P G V L H G N R | 289 |
| PSESY | G G V P G V L H G N R | 293 |
| PSEPU | G G V P G V L H G N R | 291 |
| CAUCR | G G R P G V L H G N R | 295 |
| ACICA | A G H V G V L H G N R | 289 |
| MYCTU | A G S P G A F H G S F | 297 |
| THEAQ | A G K R G V L H G S Y | 293 |
| THETH | A G K R G V L H G S Y | 293 |
| AQUAE1 | G G S V G I L H G M K | 286 |
| METTM | A G S E G V L H G S L | 278 |
| METTH | A G S E G I L H G S L | 280 |
| ARCFU | A G S K G V L H G M L | 289 |
| HALVO | T G S E G I L H G A R | 289 |
| THEMA | K G K I G Y L H G S K | 278 |
| METJA | A G E V G V L H G A K | 293 |
| SCHPO | M G K V G V F H G V R | 577 |
| YEAST | A G R P G V F H G V K | 581 |
| NEUCR | A G S K G V L H G V R | 588 |
| COPCI | M G Q P G V L H G V R | 576 |
| LACLA | K G R P G V L H G T L | 287 |
| BACSUK | K G T L G V I H G S M | 285 |
| BACSU | K G T V G V I H G S L | 285 |
| BACST | K G T K G V I H G A M | 285 |
| LACCA | R G S V G I F H G M K | 293 |
| METVO | K G E K G V L H G M L | 297 |
| CORGL | N G Q I G I L H G T R | 295 |
| BRELA | N G Q I G I L H G T R | 295 |
| **ECOLI** | **H G R V G I Y F G M K** | **283** |
| SALTY | H G R V G I Y F G M K | 283 |
| VIBPA | H G K T G I F F G M K | 282 |
| HAEIN | H G T T G I Y F G M K | 284 |
| PASMU | H G T T G I Y F G M K | 285 |
| BUCAP | H G R T G I Y F G M K | 281 |
| HELPY | K G R V G I L H G N K | 281 |
| CHLTR | T G R P G V F H G F Y | 280 |
| Consensus | **G G V L H G** | |
| ASA(TrpA2B2) | + + ± - - - - ± | |
| ASA(TrpB2) | + + ± + ± - - ± | |

**Fig. 1** Sequence of Residues β273-β283 in TrpB from *E. coli* and Comparison with 41 Other Organisms.
Consensus: residues conserved in more than 65% (17/42) of the sequences. Three Gly residues given in bold are fully conserved. ASA(TrpA2B2) and ASA(TrpB2), solvent accessible surface area of the residue side-chain in the $TrpA_2.TrpB_2$(wt, $K^+$) complex and in the isolated $TrpB_2$(wt, $K^+$) dimer from *S. typhimurium*, respectively, expressed as percentages of the ASA for the same side chain in a Gly-X-Gly context. (—), ASA ≤ 5%; (±), 20% < ASA ≤ 40%; (+), 40% < ASA. We used the structure of the $TrpB_2$ dimer in the $TrpA_2.TrpB_2$ complex as a model for the structure of the isolated $TrpB_2$ dimer, which is unknown. The ASA values were calculated from the PDB file 1TTQ (Table 1) and the sequences were retrieved and aligned as described under Materials and Methods. Other abbreviations: ACICA, *Acinetobacter calcoaceticus;* AQUAE1, from the *trpB1* gene of *Aquifex aeolicus;* ARATH1, *Arabidopsis thaliana;* ARATH2, *Arabidopsis thaliana;* ARCFU, *Archaeoglobus fulgidus;* BACST, *Bacillus stearothermophilus;* BACSU, *Bacillus subtilis;* BACSUK, *Bacillus subtilis* (strain K); BRELA, *Brevibacterium lactofermentum;* CAMAC, *Camptotheca acuminata;* CAUCR, *Caulobacter crescentus;* CHLRE, *Chlamydomonas reinhardtii;* CHLTR, *Chlamydia trachomatis* (serotype D, strain UW3/Cx); COPCI, inky cap *(Coprinus cinereus);* CORGL, *Corynebacterium glutamicum;* ECOLI, *Escherichia coli;* HAEIN, *Haemophilus influenzae* (strain Rd KW20); HALVO, *Haloferax volcanii* (strain WFD11); HELPY, *Helicobacter pylori* (strain 26695); LACCA, *Lactobacillus casei;* LACLA, *Lactococcus lactis* subsp. *lactis;* MAIZE1, maize; MAIZE2, maize; METJA, *Methanococcus jannaschii;* METTH, *Methanobacterium thermoautotrophicum* (strain Delta H); METTM, *Methanobacterium thermoautotrophicum* (strain Marburg/DSM 2133); METVO, *Methanococcus voltae;* MYCTU, *Mycobacterium tuberculosis* (strain H37RV); NEUCR, *Neurospora crassa;* PASMU, *Pasteurella multocida;* BUCAP, *Buchnera aphidicola;* PSEAE, *Pseudomonas aeruginosa;* PSEPU, *Pseudomonas putida;* PSESY, *Pseudomonas syringae;* SALTY, *Salmonella typhimurium;* SCHPO, *Schizosaccharomyces pombe* (fission yeast); SYNY3, *Synechocystis sp.* (PCC 6803); THEAQ, *Thermus aquaticus;* THEMA, *Thermotoga maritima;* THETH, *Thermus thermophilus;* VIBPA, *Vibrio parahaemolyticus;* YEAST, yeast *(Saccharomyces cerevisiae).* The sequences from *Pyrococcus horikoshii, Sulfolobus solfataricus* and the *trpB2* gene of *Aquifex aeolicus* were very different in the considered region and were not included in the alignment.

structure of the isolated $TrpB_2$ dimer, which is unknown (PDB 1TTQ, Table 1).

The side chains of residues βHis273, βArg275, βVal276 and βLys283 are accessible to the solvent both in the isolated $TrpB_2$ dimer and in the $TrpA_2.TrpB_2$ complex. Their ASA values are compatible with their high variability between organisms. The side chains of residues βIle278 and βTyr279 are accessible to the solvent in the isolated $TrpB_2$ dimer, but are buried in the $TrpA_2.TrpB_2$ complex. Their burying in the complex is compatible with their hydrophobic and conserved character. The important effects of mutation βI278A on the $SDase^-$ and $TSase^-$ activities of $TrpB_2$, and the weak effects of βI278V are compatible with the respective ASA values of the $C_{\delta 1}$-$H_3$ (65% relative ASA), $C_{\gamma 2}$-$H_3$ (25%) and $C_{\gamma 1}$-$H_2$ (15%) groups of residue βIle278. The side chains of residues βPhe280 and βMet282 are buried in the structure of the isolated $TrpB_2$ dimer. The change of βPhe280 into His in the majority of the TrpB proteins from other organisms, and the change of βMet282 into a large variety of other residues, in particular polar ones, are therefore intriguing.

## Structural Analysis

The list of the contacts that are altered by the mutations of residues β273-β283 in the structure of $TrpA_2.TrpB_2$(wt, $K^+$) is given in Table 3. The environment of these residues in the same structure is given in Figure 2. We used the structure of $TrpA_2.TrpB_2$(wt, $K^+$) to generate Table 3 and Figure 2 because we introduced the mutations in the wild-type enzyme and performed our experiments in the presence of $K^+$ ions. However, Trp-synthase is an allosteric enzyme and some of the residues that belong to segment β273-β283 or its neighborhood have different conformations and form different contacts in the available crystal structures. We therefore performed an analysis of the contacts made by residues β273-β283 in each of the structures listed in Table 1. Whatever the structure, residues β273-β283 do not belong to the binding sites of the ligands at the α- and β-sites; moreover, βTyr279 and βPhe280 are anchored in the wall of the indole tunnel. In the following paragraphs, we describe the potential structural consequences of the mutations that we constructed, and compare them to the variations of the SDase and TSase activities that we measured.

**Table 3** Contacts Altered by Mutation of Residues β273-β283 in the Structure of $TrpA_2$.$TrpB_2$(wt, $K^+$).

| Mutation | Contacts |
|---|---|
| H273A | TrpB: Ile262 (S) |
| R275A | TrpA: Asn108 (S, H-bond) |
| | TrpB: Gln288 (S), Thr289 (B, H-bond), Ala290 (B) |
| V276A | TrpB: Phe12 (S), Arg275 (B), Gly277 (B), Lys283 (S), Ala284 (B),Pro285 (S) |
| I278V | TrpA: Ala103 (S), Phe107 (S), Phe139 (S) |
| | TrpB: Tyr16 (S) |
| V278A | TrpA: Ala103 (S) |
| | TrpB: Tyr16 (S), Gly277 (B), Tyr279 (B), Met282 (B), Lys283 (S) |
| Y279F | TrpA: Ser55 (S), Asp56 (B, H-bond; S, H-bond) |
| F279L | TrpA: Phe54 (S) |
| | TrpB: Phe280 (S), Ile294 (S) |
| | HOH: 473 |
| L279A | TrpA: Phe54 (S) |
| | TrpB: Phe280 (B, S), Ile294 (S) |
| | HOH: 435, 473 |
| F280A | TrpB: Lys167 (B, S), Tyr279 (B, S), Phe306 (S), Pro307 (S) |
| A280G | TrpB: Tyr279 (B), Gly281 (B), Met282 (S) |
| G281A | TrpB: Tyr16 (B), Pro194 (S), Met282 (S) |
| | HOH: 434 |
| M282A | TrpB: Ala192 (B, S), Gly193 (B), Tyr279 (B), Phe280 (S), Gly281 (B) Lys283 (B), Ala284 (S), Phe306 (S), Ser308 (S), Pro311 (S) |
| K283A | TrpA: Phe107 (S) |
| | TrpB: Glu11 (B, H-bond; S), Tyr16 (S), Val276 (S), Ile278 (S), Ala284 (B) |

The contacts and potential H-bonds with residues of TrpA, residues of $TrpB_2$ and water molecules are indicated. They were calculated from the PDB file 1TTQ, as described under Materials and Methods.
(B): contact with a backbone atom; (S): contact with a side-chain atom; (H-bond): potential H-bond. H273A, R275A, V276A, F280A, M282A, K283A, contacts made by the chemical groups located beyond position β (*i. e.* in positions γ, δ, ...) in the side chain of the corresponding wild-type residue. I278V, V278A, contacts made by the $C_{\delta1}$-$H_3$ group, and by the $C_{\gamma1}$-$H_2$ and $C_{\gamma2}$-$H_3$ groups of βIle278 respectively. Y279F, F279L, L279A, contacts made by the $O_\eta$-H group, by the $C_\zeta$, $C_{\varepsilon1}$-H and $C_{\varepsilon2}$-H groups, and by the $C_{\delta1}$-H, $C_{\delta2}$-H and $C_\gamma$ groups of βTyr279 respectively. A280G, contacts made by the $C_\beta$-$H_2$ group of βPhe280. G281A, contacts made by the $C_\alpha$-$H_2$ group of βGly281. The contacts with the residues of $TrpB_2$ have been reported previously (Rondard and Bedouelle, 1998).

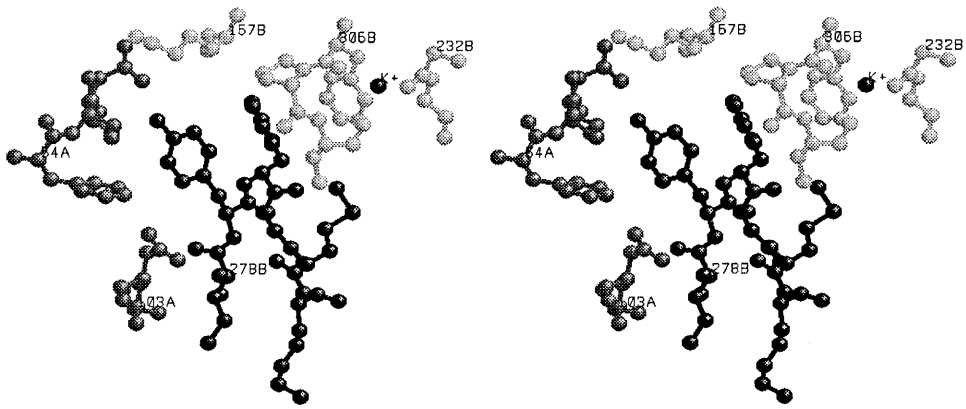


**Fig. 2** Environment of Residues β278-β282 in the Structure of $TrpA_2$.$TrpB_2$(wt, $K^+$) (Stereo View).
The $K^+$ ion and residues β278-β282 are in black, the other residues of TrpB (β167, β232-β233, β306-β308) in light grey, and the residues of TrpA (α54-α56, α103-α104) in dark grey. The Figure was drawn from the PDB file 1TTQ (Table 1).

### βIle278

The mutation βI278V had no effect on the activities of $TrpB_2$, whereas βI278A had strong effects. Therefore, the effects of βI278A were due to the deletions of the $C_{\gamma1}$-$H_2$ and $C_{\gamma2}$-$H_3$ groups of residue βIle278 and not to the deletion of its $C_{\delta1}$-$H_3$. The $C_{\gamma1}$-$H_2$ and $C_{\gamma2}$-$H_3$ groups of βIle278 contact the backbone of βGly277, βTyr279 and βMet282, and the side chains of βTyr16 and βLys283. These con-

tacts vary little between the different structures of Trp-synthase (Tables 1 and 3). The effects of βI278A on the activities of $TrpB_2$ occurred necessarily through these contacting residues. Residue βTyr16 is involved neither in the binding of the substrates nor in the allosteric communication between the α- and β-sites (Bahar and Jernigan, 1999; Schneider *et al.,* 1998). The mutations βY279A and βK283A had only weak effects on the activities of $TrpB_2$, whereas βM282A strongly affected them and belonged to the same mutational class as βI278A (Table 2). Therefore, βI278A could exert its effects through residue βMet282. Mutation βI278A abolishes several contacts of residue βIle278 with residues αAla103, αPhe107 and αPhe139 of TrpA. The properties of the mutant $TrpB_2$(βI278A) showed that these contacts are not necessary for the activation of $TrpB_2$ by TrpA.

### βTyr279

The mutation βY279L had a smaller effect on the $SDase^-$ activity of $TrpB_2$ than βY279F and yet it deleted more of the side chain of residue βTyr279. Therefore, either the mutation βY279F resulted in an unfavorable conformational change of $TrpB_2$ that did not exist for βY279L, or βY279L resulted in a favorable conformational change. The observation that Leu is the preferred side chain in position β279 (67% of the sequences) is in favor of the second hypothesis. The variations of free energy of interaction between monoclonal antibody mAb164 and the isolated $TrpB_2$ dimer (its antigen), due to the mutations βY279F, βY279L and βY279A are equal to 0.3, 2.7 and 4.4 kcal/mol respectively (Rondard and Bedouelle, 1998). Their values show that mAb164 does not detect a conformational change in its epitope (residues β276-β283), either unfavorable and due to βY279F or favorable and due to βY279L. Therefore, if it exists, this conformational change involves residues of $TrpB_2$ located outside of the mAb164 epitope. The mutation βY279L decreased the $TSase^-$ activity of $TrpB_2$ more than its $SDase^-$ activity. Residue βTyr279 is anchored in the wall of the indole tunnel, and its side chain is accessible to the solvent in the isolated $TrpB_2$ dimer (Figure 1). It therefore caps the portion of the indole tunnel which is included in the isolated $TrpB_2$ dimer. The change of βTyr279 into the more hydrophobic Leu residue could thus perturb the entrance of indole from the aqueous medium into this portion of the tunnel. The activation of $TrpB_2$ by TrpA was higher for the βY279F, βY279L and βY279A mutants of $TrpB_2$ than for the wild type (Table 2). Therefore, the interactions between the side chain of βTyr279 and residues α54 to α58 of TrpA, observed in 11/13 structures of Trp-synthase (all except 1BKS and 2WSY; Table 1), were unnecessary or even detrimental to this activation.

### βPhe280

The mutations βF280A and βF280G strongly decreased the $SDase^-$ activity of $TrpB_2$ and less strongly its $TSase^-$ activity. Mutation βF280A also decreased the activation of $TrpB_2$ by TrpA. The aromatic ring of residue βPhe280, deleted by mutations βF280A and βF280G, interacts with the COMM domain in 11/13 structures of Trp-synthase (all except 1TTP and 1A5A; Table 1). These interactions occur either with residue βLys167, or with βCys170, βLeu174, βTyr186 and βLeu188. The aromatic ring of βPhe280 also interacts with βPhe306 in 10/13 structures (all except 1BKS, 2TSY and 2TRS; Table 1). Thus, βPhe280 communicates with the β-site by the intermediate of the COMM domain and of the cation binding loop (residues β304-β308), which could explain the effects of mutations βF280A and βF280G on the $SDase^-$ activity of $TrpB_2$. It also communicates with TrpA by the intermediate of the COMM domain, which could explain the effect of mutation βF280A on the activation of $TrpB_2$ by TrpA. Residue βPhe280 is anchored in the wall of the indole tunnel. Mutations βF280A and βF280G, by deleting the side chain of βPhe280, could facilitate the transport of indole through its tunnel, as previously suggested for mutations βF280S and βF280C (Ruvinov *et al.,* 1995). This opening of the indole tunnel by βF280A and βF280G, and the fact that L-Ser, the substrate of the $SDase^-$ reaction, accesses the β-site directly from the solvent (Hyde *et al.,* 1998), could explain why these two mutations affected more strongly the $SDase^-$ than the $TSase^-$ activity of the isolated $TrpB_2$ subunit. The properties of βF280A and βF280G suggest that indole accesses the β-site through its tunnel in the isolated $TrpB_2$ dimer as in the $TrpA_2.TrpB_2$ complex, and not directly from the solvent.

The mutation βF280G decreased the $TSase^-$ activity of $TrpB_2$ more than βF280A. Moreover, βF280G increased the activation factor of $TrpB_2$ by TrpA whereas βF280A decreased it. The $C_\beta$-$H_2$ group of residue βPhe280, which is deleted by βF280G but not by βF280A, contacts the side chain of βMet282 in 3 out of 4 structures of $TrpA_2.TrpB_2$(wt, $K^+$) (all except 1A5A; Table 1). Thus, the different properties of the mutations βF280G and βF280A could be mediated by residue βMet282. Alternatively, they could result from a conformational change around residue β280, due to its mutation into the flexible Gly residue.

### βMet282

The side chain of residue βMet282 contacts βPhe306 in eight out of thirteen structures of Trp-synthase (all except 2TYS, 2TSY, 2TRS, 1TTP and 2WSY; Table 1) and forms close contacts with βSer308 in all the structures. Thus, βMet282 communicates with the β-site through the cation binding loop, which could explain the effects of mutation βM282A on the $SDase^-$ and $TSase^-$ activities of $TrpB_2$. As mentioned above, the residue in position β282 is Met every time that the residue in position β280 is Phe. The only interaction between the side chains of the two residues occurs between the $C_\beta$-$H_2$ of βPhe280 and the $C_\varepsilon$-$H_3$ of βMet282, in the structures 1TTQ, 1BEU and 1A5B (Table 1). This interaction, together with the hydrophobic character of the Phe and Met residues, might be related to the residue co-variation.

### Mutations into Proline

The ($\phi$, $\psi$) dihedral angles of a proline residue take values that are defined and different from those of residues βTyr279, βPhe280 and βMet282 in the structures of Trp-synthase (Table 1; MacArthur and Thornton, 1991). Therefore, mutations βY279P, βF280P and βM282P necessarily changed the conformation of the polypeptide backbone of $TrpB_2$. Accordingly, these mutations strongly affected the recognition of the $TrpB_2$ dimer by antibody mAb164 (Rondard and Bedouelle, 1998). Here, we found that they strongly affected the $SDase^-$ and $TSase^-$ activities of $TrpB_2$. The mutation βY279P had effects similar to those of βF280A on these activities. These effects were different from those of βY279F, βY279L and βY279A. Mutation βY279P could thus act indirectly, through a conformational change of residue βPhe280. Mutation βF280P had effects similar to those of βI278A, βF280G and βM282A. βF280P could thus act directly, by changing the side chain of residue βPhe280. Among all the mutations that we studied βM282P had the strongest effects. These effects were qualitatively similar to those of βM282A, except that βM282P decreased more the $TSase^-$ activity of $TrpB_2$ than its $SDase^-$ activity, as βY279L, whereas βM282A decreased them equally. The properties of mutation βM282P suggested that it could act both directly by changing the side chain of residue βMet282, and indirectly through a conformational change of βTyr279.

## Conclusions

The mutations of residues β273-β283 could be grouped into several classes, according to their effects on the serine deaminase activity of the isolated $TrpB_2$ dimer, on its Trp-synthase activity, and on the activation of the β-reaction by TrpA. The mutations of residues βIle278, βPhe280 and βMet282 were the most detrimental. In general the mutations into Pro, which changed the conformation of residues β278-β282, had stronger effects than deletions of side chains groups. The comparison of mutations βF280A and βF280G indicated that the $C_\beta$-$H_2$ group of residue βPhe280 plays an important structural and functional role. The different classes of mutations and an analysis of the crystal structures showed the existence of an indirect communication between residues β278-β282 and the β-site.

The mutations βY279P, βF280A and βF280G decreased more the $SDase^-$ activity of $TrpB_2$ than its $TSase^-$ activity, whereas βY279L decreased more the $TSase^-$ activity than the $SDase^-$ activity. It has been proposed that residus βTyr279 and βPhe280 serve as a molecular gate for the transfer of indole from the α-site to the β-site through its tunnel (Hyde *et al.,* 1988). It would thus be interesting to test if βY279L slows down the transport of indole through its tunnel and if βF280A and βF280G speed it up, as shown for the mutations βF280C and βF280S using the binding of Nile red and rapid kinetics experiments (Anderson *et al.,* 1991, 1995; Ruvinov *et al.,* 1995). These experiments could be done both for the isolated $TrpB_2$ subunit and for the $TrpA_2$.$TrpB_2$ complex. Only two mutations, βY279P and βF280A, decreased the factor by which TrpA activated the β-reaction. They also decreased the $SDase^-$ activity of $TrpB_2$ and left its $TSase^-$ activity unchanged. These two mutations suggest the existence of a link between the activation by TrpA and the transport of indole.

The most frequent sequence for residues β278-β282 in Trp-synthases is 278-VLHGX-282 where X can be any of 7 residues. The Trp-synthases from *E. coli* and *S. typhimurium,* which carry the sequence 278-IYFGM-282, thus belong to a minority group. It would be interesting to reconstitute the corresponding evolutionary intermediates in the *E. coli* or *S. typhimurium* Trp-synthase.

Our mutational scanning of residues β273-β283 of TrpB allowed us to identify the functionnally important residues of this segment. The most interesting mutants should be characterized by rapid kinetics and X-ray crystallography. The corresponding mutations could be useful to deepen our understanding of the substrate channeling, of the allosteric communication between distant active sites, and of the relationships between these two phenomena in one of the experimental systems which has been the most thoroughly studied.

## Materials and Methods

### Enzyme Assays

The wild-type TrpA protein and the wild-type and mutant $TrpB_2$ dimers were purified as described (Rondard and Bedouelle, 1998). The synthesis of tryptophan from indole and L-Ser was monitored with $A_{289nm}$, as described previously (Faeder and Hammes, 1970). The reaction mixture contained $5.10^{-4}$ M indole, $5.10^{-2}$ M L-Ser, $5.10^{-5}$ M PLP, 0.1 mg/ml bovine serum albumin, 0.1 M potassium phosphate buffer, pH 7.8. The serine deaminase reaction was monitored with $A_{340nm}$ as described (Crawford and Ito, 1964). The reaction mixture contained 0.1 M L-Ser, $5.10^{-5}$ M PLP, $1.10^{-2}$ M NADH, 0.1 mg/ml bovine serum albumin, 0.1 M potassium phosphate buffer, pH 7.8, and 10 μg/ml lactate dehydrogenase (type II, Sigma). The reactions were performed at 25 °C, either in the absence or in the presence of a 6- to 15-fold molar excess of TrpA over TrpB. One unit of activity corresponds to the formation of 0.1 μmol of L-Trp or the disappearance of 0.1 μmol NADH in 20 min. Specific activity is expressed as the number of units per milligram of protein.

### Analysis of the Sequences and Structures

The sequences of the TrpB proteins from various organisms were retrieved by using the DBGET database (Kanehisa, 1997; http://www.genome.ad.jp/dbget-bin/www_bfind?protein-today) and by entering the key-word 'tryptophan synthase' into the search box in 'bfind' mode. The sequences were aligned with the Clustal W program (version 1.74; Thompson *et al.,* 1994). The structures of the *S. typhimurium* Trp-synthase were analyzed with the WHAT IF program as described earlier (Rondard and Bedouelle, 1998).

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