The αβ2 tryptophan synthase complex is a model enzyme for understanding allosteric regulation. We report the functional and regulatory properties of the βS178P mutant. Ser-178 is located at the end of helix 6 of the β subunit, belonging to the domain involved in intersubunit signaling. The carbonyl group of βSer-178 is hydrogen bonded to Gly-181 of loop 6 of the α subunit only when a subunit ligands are bound. An analysis by molecular modeling of the structural effects caused by the βS178P mutation suggests that the hydrogen bond involving αGly-181 is disrupted as a result of localized structural perturbations. The ratio of α to β subunit concentrations was calculated to be 0.7, as for the wild type, indicating the maintenance of a tight αβ complex. Both the activity of the α subunit and the inhibitory effect of the α subunit ligands indole-3-acetylglycine and D,L-α-glycerol-3-phosphate were found to be the same for the mutant and wild type enzyme, whereas the β subunit activity of the mutant exhibited a 2-fold decrease. In striking contrast to that observed for the wild type, the allosteric effectors indole-3-acetylglycine and D,L-α-glycerol-3-phosphate do not affect the β activity. Accordingly, the distribution of L-serine intermediates at the β-site, dominated by the α-aminoacylate, is only slightly influenced by α subunit ligands. Binding of sodium ions is weaker in the mutant than in the wild type and leads to a limited increase of the amount of the external aldime intermediate, even at high pH, whereas binding of cesium ions exhibits the same affinity and effects as in the wild type, leading to an increase of the α-aminoacylate tautomer absorbing at 450 nm. Crystals of the βS178P mutant were grown, and their functional and regulatory properties were investigated by polarized absorption microspectrophotometry. These findings indicate that (i) the reciprocal activation of the α and β activity in the αβ2 complex with respect to the isolated subunits results from interactions that involve residues different from βSer-178 and (ii) βSer-178 is a critical residue in ligand-triggered signals between α and β active sites.

Allosteric proteins are key elements of cellular regulation, because they allow for a fine adaptation of the living organisms to changes in metabolite concentration and physico-chemical conditions. Tryptophan synthase is an allosteric enzyme, composed of two α and two β subunits, catalyzing the last two steps in the biosynthesis of L-tryptophan (1–3) (Fig. 1). The α active site cleaves indole-3-glycerol phosphate into indole and D-glyceraldehyde-3-phosphate. The β active site contains a pyridoxal 5′-phosphate molecule bound to Lys-87 via a Schiff base and catalyzes the replacement of the β-hydroxyl of L-serine with indole. The latter compound is intramolecularly channeled from the α site via a hydrophobic tunnel connecting the α and β active sites, as proposed on the basis of the first crystal structure of the enzyme (4). The α and β activities are reciprocally modulated. Extensive functional and structural studies of the wild type enzyme and several mutants have unveiled some of the mechanisms underlying catalysis and allosteric regulation (3). In particular, the β subunit exists either in a closed, catalytically active state, when the α-aminoacylate is the most populated catalytic intermediate, or in an open, catalytically less active state, when the external aldime is the predominant species (5–8). When the former intermediate is formed at the β active site, signals are generated that trigger the activation of the α active site, keeping in phase the activation of the two sites. Intersubunit communications are mediated by a movable domain of the β subunit, called the COMM (communication) domain, interacting with the mobile loops 2 and 6 of the α subunit (9, 10). By comparing the three-dimensional structure of the enzyme in the presence and absence of the α subunit ligand 5-fluorooridole-3-propanol phosphate and the α-aminoacylate species (10), it was noted that six new polar interactions are formed, five of which are between residues of α loop 2 and β helix 6, and only one is between αGly-181 of loop 6 and βSer-178 of helix 6. Whether there is a unique pathway or multiple specialized pathways of intersubunit communication is not yet known. In fact, the subunit interface mediates i) the 10–100-fold activation of the α and β activity in the αβ2 complex with respect to the isolated subunits, ii) the reciprocal action of α and β subunit ligands on kinetic, thermodynamic, and dynamic properties of the opposite active sites, and iii) pH and monovalent cation modulation of β subunit activity.

To gain insight into the role of βSer-178-αGly-181 interaction in regulating the allosteric communication, βSer-178 was mutated to a proline residue. The rationale for the choice of

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1 The abbreviations used are: COMM, communication; bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; MOPS, 3-(N-morpholino)propanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; PCR, polymerase chain reaction; IAG, indole-3-acetylglucose; GP, D,L-α-glycerosephosphate.
proline was that the interaction between βSer-178 and αGly-181 is mediated by a hydrogen bond between the carbonyl oxygen of βSer-178 and the amidic nitrogen of αGly-181. βSer-178 is not a conserved residue, as demonstrated by a multiple alignment of the amino acid sequence of β subunits from different sources.\(^2\) Therefore, proline mutation was chosen for its unique feature of preventing the formation of the hydrogen bond with αGly-181.\(^3\) Furthermore, to obtain structure and function relationships, crystals of the mutant were grown, and the catalytic competence of the mutant was investigated by polarized absorption microspectrophotometry.

**MATERIALS AND METHODS**

**Reagents**—All chemicals, obtained from Sigma, except cesium chloride (Roche Molecular Biochemicals), were of the best available quality and were used without further purification. Glyceraldehyde-3-phosphate was obtained from the monobarium salt of the diethyl acetal form, and its concentration was determined using an assay based on the activity of glyceraldehyde-3-phosphate dehydrogenase. The stock solution was kept at pH <2 and stored at −20 °C. Oligonucleotides were from Eurogentec. Restriction enzymes and the agarose gel extraction kit were from Roche Molecular Biochemicals. Ligation was performed by using a DNA ligation system from Amersham Pharmacia Biotech.

**α and β Activity Assays**—The β activity assay was carried out as previously described (11). The buffer for the assay was stored at −20 °C, kept in the dark, and used within 15 days from preparation. The assay for the α subunit reverse activity was carried out in 50 mM bis-Tris propane, 0.2 mM indole, 0.04 mM pyridoxal 5'-phosphate, and 0.5 mM glyceraldehyde-3-phosphate, pH 7.5, at 20 °C. The assay solution was prepared immediately before use and kept at 4 °C. Measurements were terminated within 1 h from solution preparation. Activity assays were carried out using 1-mm path length quartz cuvettes.

**Spectrophotometric and Spectrofluorimetric Measurements**—Measurements were carried out in a solution containing 25 mM bis-Tris propane in the absence and presence of 50 mM L-serine at 20 °C. The final pH was adjusted with concentrated HCl. pH measurements were performed with a Radiometer pHM83 pH meter, equipped with an Ingold-Mettler Lot406-M3 microelectrode. The effect of pH on the equilibrium distribution of catalytic intermediates was measured in a solution containing 50 mM MOPS, 50 mM Bicine, 50 mM proline, and 1 mM EDTA (MBP buffer). The pH was raised to 11.5 with concentrated sodium hydroxide and then back-titrated with HCl to the desired pH to keep constant the ionic strength of the buffer in the pH range 6–11. Spectrophotometric measurements were carried out using a Varian Cary 400. Spectrofluorimetric measurements were carried out using a PerkinElmer LS50B. Data were analyzed with the software SigmaPlot 6.0 (SPSS Science).

**Computational Methods**—Molecular simulations of the site-directed mutagenesis were carried out using SYBYL version 6.5 (Tripos Associates). The Tripos force field was used throughout the calculations. The Ser to Pro mutation was carried out using the Biopolymer option on the tryptophan synthase structure with 5-fluoroorindole-3-propanol phosphate bound to the α-subunit, Protein Data Bank file 1a50.pdb (10). Charges were assigned according to Gasteiger-Huckel, and water molecules present in the Protein Data Bank file were included in the calculations. A first minimization was performed on the mutated amino acid to reduce negative contacts with neighboring residues, and then a second minimization was performed on the α/β interface of the enzyme. The radius of the latter minimized region was 10 Å, centered at the mutation site when all interactions were considered, and 12 Å when only steric interactions were considered. The remaining portion of the protein was kept rigid. The minimizations were performed according to the Powell method. The resulting structure was superimposed on the wild type for evaluating structural changes upon mutation.

**Preparation of the βS178P Mutant**—The bacterial strain Escherichia coli CB149 carrying the plasmid pEBA10 for the expression of tryptophan synthase complex from Salmonella typhimurium was kindly provided by Dr. E. W. Miles, National Institutes of Health. Site-directed mutagenesis was performed by overlap extension PCR (12). External primers annealing over the 5'- and 3'-terminal sequences of the trpB gene in the pEBA10 plasmid were PE1 and PE2, respectively, as described by Yang et al. (13). Mutagenic primers for βS178P were 5'-CGTAACTACCGGCCAGTCGC-3' and its complementary sequence (S178Prev and S178Pfrw, respectively). PCR was performed at 95 °C for 50 s, 62 °C for 50 s, and 74 °C for 60 s, with 2.5 units of Vent polymerase, using plasmid pEBA10 as template. The 570- and 810-base-
pair products from the first PCR reaction were used as templates in a second PCR with the external primers PE1 and PE2 to generate the complete coding sequence of **trpB**. The 1100-base pair **BgIIBXhoI** fragment generated from the second PCR was subcloned into pEBA1 and digested with the same restriction enzymes. The newly generated plasmid was used to transform **E. coli JM109** and then **E. coli CB149**. The **trpB** gene carrying the mutation **S178P** was fully sequenced. Sequence analysis showed the presence of the correct **S178P** mutation, as well as an Arg to Ser mutation at position 34 of the amino acid sequence. This mutation was already present in the original plasmid.\(^4\)

**Purification of the **\(\beta S178P\)** Mutant**—The growth of the bacterial strain **E. coli CB149** containing the plasmid pEBA10, with the mutation **S178P** in the **trpB** gene, of the **S. typhimurium** tryptophan synthase was carried out according to Yang et al. (13). Purification of the mutant was carried out as described for the wild type (13). Enzyme purity was carried out according to Yang et al. (13). Enzyme purity was estimated on the basis of the absorption intensity at 280 nm to remove monovalent cations and stored at 80 °C. Protein concentration was estimated on the basis of the absorption intensity at 280 nm (14).

**Crystallization of **\(\beta S178P\)** Tryptophan Synthase**—Crystals of the mutant were grown according to Schneider et al. (10). The mutant was crystallized using the hanging drop method by mixing 5 µl of a solution containing 10 mg/ml enzyme, 50 mM Bicine, pH 7.8, with an equal quantity of reservoir solution containing 50 mM Bicine, 1 mM EDTA, 12% polyethylene glycol 8000, and 1.4 mM spermine, pH 7.8, at 21 °C. Monoclinic crystals grew within a few days and were stored in a solution containing 50 mM Bicine, 1 mM EDTA, and 20% polyethylene glycol 8000, at 4 °C.

**Microspectrophotometric Measurements**—Single crystals of **\(\beta S178P\)** tryptophan synthase were suspended in a solution containing 20% polyethylene glycol 8000, 50 mM bis-Tris propane, pH 7.8, and mounted in a quartz flow cell. Replacement of the suspending medium was carried out by passing solutions through the cell. The cell was placed on the stage of a Zeiss MPM03 microspectrophotometer, equipped with a 10 × Zeiss UV-visible ultrafluar objective (15, 16). Polarized absorption spectra were collected between 315 and 550 nm with the electric vector of the linearly polarized light parallel to the extinction directions on the (210) flat face of monoclinic crystals. All experiments were carried out at 20 °C.

### RESULTS

Because the three-dimensional structure of the **\(\beta S178P\)** mutant tryptophan synthase was not available, a molecular model was created to evaluate the effects of the mutation on the protein conformation. The model was obtained by changing **\(\beta S178\)** into a proline in the wild type crystallographic structure and performing two energy minimizations of the resulting structure. The comparison of the wild type and mutant (Fig. 2) indicates that the mutation causes only localized structural changes, leading to the loss of the hydrogen bond between **\(\beta S178\)** and **αGly-181**. No other significant movements appear to take place either in the **α** or **β** subunit. An experimental evaluation of the effect of mutation on the strength of the **α**−**β** association was obtained by the quantitative densitometric analysis of the stained bands in the electrophoretic pattern of the purified mutant and wild type protein under denaturing conditions (data not shown). It was found that the ratio of **α** and **β** subunit concentrations is 0.7, as for the wild type (17), indicating that the mutation does not appreciably affect the inter-subunit affinity of the **αβ** mutant complex.

The **α** subunit activity of the mutant was found to be very similar to that of the wild type, and the **β** subunit activity was about 2-fold lower. Furthermore, the **α** subunit activity of the mutant was inhibited by the binding of IAG and GP (Fig. 3, a and b), as previously observed for the wild type enzyme (18). The calculated inhibition constants of IAG and GP for the mutant, assuming a single isotherm of binding, are 0.36 ± 0.04 and 12.7 ± 2.9 mM, respectively. The same value was determined for GP in the wild type, and a 3-fold higher value was found for IAG (18). These findings indicate that the mutation does not significantly affect the **α** active site geometry and does not impair the binding of a subunit ligands. It should be noted that the activity of the mutant at infinite ligand concentrations is not fully abolished, whereas it is fully abolished for the wild type. This finding might be associated with different types of inhibition, as previously observed for indole-3-propanol phosphate, competitive with respect to glyceraldehyde-3-phosphate and noncompetitive with respect to indole (19). In contrast to that observed for the wild type, no effect of IAG (18) and GP (17) on **β** subunit activity was detected (Fig. 3, c and d), indicating that this feature of the allosteric regulation between **α** and **β** subunits is lost in the **βS178P** mutant.

A well characterized effect of the **α**−**β** allosteric regulation is the change of the equilibrium distribution of catalytic intermediates at the **β** active site caused by a subunit ligands (2, 5, 8, 15, 16, 20). This distribution is also affected by pH, monovalent cations, and temperature (8, 21). Specifically, **α** subunit ligands, low pH, cesium ions, and high temperature stabilize the **α**-aminoacrylate intermediate, whereas high pH, sodium ions, and low temperature stabilize the external aldimine. Moreover, high pH and monovalent cations favor the accumulation of a quinonoid intermediate (16). To further evaluate the effect of mutation on the **α**−**β** regulation, the influence of **α** subunit ligands, monovalent cations, and pH on the equilibrium distribution of catalytic intermediates of the **β** active site was investigated. The absorption spectrum of the mutant enzyme in the absence of ligands (Fig. 4a) exhibits a typical band at 412 nm, assigned to the ketoenimine form of the internal aldimine. The second absorption band at about 330 nm, assigned to the enolimine form of the internal aldimine, is more intense than in

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\(^4\) E. W. Miles, personal communication.
Allosteric Regulation of Tryptophan Synthase

The equilibrium distribution of intermediates for the mutant (21) indicates a negligible influence of IAG and GP on the rate of \( \alpha \) and \( \beta \) reactions catalyzed by the wild type tryptophan synthase. Activities in the presence of ligands are calculated as a percent of the \( \alpha_{\beta} \) complex activity in the absence of ligands for the mutant (O) and wild type enzyme (C). Dependence of the relative specific activity on IAG (a) and GP concentrations (b) is shown. For the mutant, the dissociation constants, calculated by fitting data to a binding isotherm, are 0.36 ± 0.04 and 12.7 ± 2.9 mM for IAG and GP, respectively. For the wild type, the dissociation constants are 0.99 ± 0.28 and 11.4 ± 4.4 mM for IAG and GP, respectively. For the mutant, the percent residual activity at infinite IAG and GP concentrations is 6.2 ± 3 and 16.8 ± 5.8, respectively. For the wild type, the activity at infinite IAG and GP concentrations is fully abolished. The \( \alpha \) activity of the wild type and mutant is 6000 and 5900 units/mg, respectively. Dependence of the \( \beta \) activity on IAG (c) and GP concentrations (d) is shown. The \( \beta \) activity of the wild type and the mutant is 1168 and 504 units/mg, respectively.

Spectra were recorded in the absence ( — ) and presence of 1.6 mM IAG or 50 mM GP ( — — — ). GP is a disodium salt.

Effect of IAG and GP on enzyme activity. The spectrum was recorded on a solution containing 0.77 mg/ml mutant enzyme, 25 mM bis-Tris propane, pH 7.8, at 20 °C. a, absorption spectrum of the wild type tryptophan synthase. Absorption at 450–470 nm, as observed in O-acetylserine sulfhydrylase (22) and cystathionine \( \beta \)-synthase (23, 24). This result indicates a negligible influence of IAG and GP on the equilibrium distribution of \( \beta \)-intermediates. The same negligible effect was detected by monitoring the change of the fluorescence emission at 500 nm upon excitation at 420 nm (data not shown), typical of the external aldimine, the only highly fluorescent species of tryptophan synthase (25).

Sodium ions strongly favor the accumulation of the external aldimine species of tryptophan synthase (21). This behavior was attributed to the stabilization of a partially open conformation of the enzyme (21, 26). Binding of sodium ions to the mutant causes a very small increase of the absorption band at 422 nm, assigned to the external aldimine (data not shown). Accordingly, the fluorescence emission at 500 nm is enhanced (Fig. 5a). The dissociation constant of sodium ions was found to be 153 mM (Fig. 5b), a value significantly higher than the dissociation constants of 1 and 19 mM previously determined for the wild type (21). IAG and GP do not significantly perturb the equilibrium distribution of intermediates also in the presence of saturating concentrations of sodium ions (data not shown).

The equilibrium distribution of intermediates for the mutant enzyme in the presence of sodium ions is affected by pH (Fig. 6), in contrast to that observed for the wild type. Furthermore, the
To correlate structural, regulatory, and functional information, crystals of βS178P tryptophan synthase were grown. The crystallization conditions were very similar to those previously used to obtain crystals of the wild type (10). The functional and regulatory properties of βS178P crystals were investigated by polarized absorption microspectrophotometry. Polarized absorption spectra of mutant tryptophan synthase crystals were recorded in the presence of L-serine, with and without sodium ions and with and without IAG (Fig. 7). When L-serine is added to the mutant tryptophan synthase crystals in the absence of cations, the α-aminoacrylate is the predominant species, as in the wild type. The presence of sodium ions causes a significant shift of the equilibrium distribution of intermediates, favoring the accumulation of the external aldimine at a higher concentration than in solution (Fig. 5) but lower than in the wild type enzyme (21). Saturating concentrations of IAG cause only a small shift of the equilibrium toward the α-aminoacrylate species, indicating that the loss of communication between α and β subunits is detectable also in the crystalline enzyme.

**DISCUSSION**

A model of the structural pathway for the transmission of allosteric signals between α and β subunits of tryptophan synthase was proposed on the basis of fluorescence and phosphorescence properties of βTrp-177 in the absence and presence of α subunit ligands (29, 30). This model was later validated by the crystallographic structures of the enzyme (9, 10, 31–34) and by computational methods (35). Key structural elements of the allosteric communication are loops 2 and 6 of the α subunit and the β helix 6 in the COMM domain (9, 10, 33, 34). The βSer-178 residue is adjacent to βTrp-177 and is the last amino acid of the β helix 6. Crystallographic studies of 5-fluoroorindole-3-propanol phosphate (10) and indole-3-propanol phosphate enzyme complexes (34) showed that the loop 6 of the α subunit interacts with β helix 6 via a hydrogen bond between the amide nitrogen of αGly-181 and the carbonyl oxygen of βSer-178. The bond length is 3.4 and 2.8 Å, respectively. A value of 3.9 Å was found in the GP-enzyme complex (9), indicating that bond formation is facilitated by the indole ring of the α subunit ligands interacting with αGly-184 and causing a motion of the α loop 6. In turn, a movement of the entire α loop 2 and, in particular, the displacement of the active site residue αAsp-60 are triggered by the relocation of αThr-182. Thus, new polar interactions are formed between α loop 2 and β helix 6 with the transmission of signals to the COMM domain (10). A theoretical investigation indicated that motions of the COMM domain are linked to the motions of residues β174–179, localized at the entrance of the tunnel (35). Thus, the intramolecular transfer of indole from α to β active sites was proposed to be allosterically controlled via the modulation of tunnel accessibility. Recent structural studies of βA169L/βC170W mutants have indicated that residue β170 is critical in impairing channeling, whereas residue β169...
is relevant for a correct conformation of the COMM domain (36). The catalytic action of the β subunit is allosterically regulated via i) an open-closed transition of α and β subunits, ii) a change of conformational flexibility of the β subunit, iii) a pH dependence of the equilibrium distribution of intermediates and the catalytic activity, iv) a distinct influence of the monovalent cations sodium, potassium, and cesium, competitively bound to the same site of the β subunit, on the activity and distribution of intermediates, and v) effects triggered by binding of α subunit ligands.

The work on the βS178P mutant was aimed at understanding whether the pathway of communication between α and β subunits is unique or whether there are multiple, specialized pathways, each one predominantly involved in controlling a specific dynamic and functional effect on the β active site. Modelling and experimental data indicate that the replacement of βSer-178 with Pro does not apparently perturb the overall structure of the enzyme and the catalytic activity of the individual subunits within a tight tetrameric complex. Moreover, the α subunit of the mutant enzyme binds ligands with similar affinities as the wild type, indicating that the mutation does not alter the α active site. The major effect of the mutation is the loss of the ability of the α subunit ligands to affect both the β subunit activity and the equilibrium distribution of intermediates. The loss of the influence of the α subunit ligands on the β subunit is also present in three other mutants of the α subunit, i.e., D56A and P57A mutants and the presence of an α subunit ligand enhances the β activity (17, 40). Furthermore, it was shown that the loop 2 interacts only with α subunit ligands that possess an indole ring (9). On the basis of the above considerations, it is possible to propose the pathways of allosteric communication from the α subunit to the β subunit shown in Scheme 1.

When a ligand binds to the α subunit, an interaction takes place with αGly-184 in the loop 6 of the α subunit. The movement of the loop 6 is propagated to αAsp-60 via the hydrogen bond with αThr-183. This displacement allows Asp-60 to assume the correct position for catalysis. In agreement with this hypothesis, it was found that αT183A and Δα183–185 mutants are completely inactive (37). Furthermore, the movement of the α loop 6 causes the formation of a hydrogen bond between αGly-181 and βSer-178 and a shift of the β helix 6 of the COMM domain. The relocation of the β helix 6 leads to new interactions between the α loop 2 and the COMM domain, with the formation of polar interactions between αAsp-56 and βLys-167. Therefore, α loop 6 is critical for the transmission of the allosteric signals originated from α subunit ligands, whereas α loop 2 is critical for intersubunit interactions that increase the activity of both the α and β subunits in the tetramer with respect to the isolated subunits.

Another important aspect of the regulatory machinery of tryptophan synthase is the influence of monovalent cations. The binding site for monovalent cations was structurally characterized (9, 10, 31–34). The influence of monovalent cations on the external aldimine/α-aminoacrylate distribution was reported (21, 26). Na+ stabilizes the allosterically silent external aldimine species. This cation is lost after indole-3-glycerol phosphate binding, following the COMM domain displacement and the formation of the salt bridge between βLys-167 and αAsp-56 (31, 34). Furthermore, the dissociation of Na+ appears to determine the movement of two residues, βTyr-279 and βPhe-280, out of the indole tunnel (31). On the other hand, Cs+ binding leads to the accumulation of a tautomeric form of the α-aminoacrylate characterized by an absorption peak at 450 nm (21). In the absence of cations, the α-aminoacrylate tautomer absorbing at 350 nm is the predominant species (21). In the βS178P mutant tryptophan synthase the effect of Cs+ is comparable with that on the wild type enzyme. Cs+ binds to the tryptophan synthase with an apparent Kd similar to that of the wild type (21). The behavior of Na+ is very different. The affinity of this cation for the mutant is extremely reduced with respect to the wild type. This might be due to the breaking of the contact between αGly-181 and βSer-178, which, in turn, via the movement of β helix 6, stabilizes the aminoacrylate conformation, characterized by a low affinity for Na+. The pH dependence of the equilibrium distribution of catalytic intermediates is also altered in the mutant, destabilizing the external aldimine. These findings confirm the relevance of α loop 6 in the control of the equilibrium between active and inactive forms of the enzyme, i.e., between the inactive conformation associated with the external aldimine and the active conformation associated with the α-aminoacrylate. In the crystalline state the effect of Na+ is still detectable, probably because the lattice forces prevent the complete displacement of the COMM domain both in the wild type tryptophan synthase and in the mutant enzyme. As a consequence, high concentrations of Na+ influence the equilibrium distribution of intermediates and
favor the formation of the external aldimine, although not to the same extent as in the wild type. Nevertheless, the allosteric communication is lost also in the crystalline enzyme, strongly supporting the relevant role of βSer-178 in a ligand-triggered intersubunit signals and in the stabilization of the α-aminoacrylate conformation. These findings open the way to the structural characterization of an active but allosterically “knocked out” enzyme.

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