Amino Acids (2011) 41:1247–1256
DOI 10.1007/s00726-010-0802-1

ORIGINAL ARTICLE

Stereospecificity of isotopic exchange of C-α-protons of glycine catalyzed by three PLP-dependent lyases: the unusual case of tyrosine phenol-lyase

Vitalia V. Koulikova · Lyudmila N. Zakomirdina · Olga I. Gogoleva · Marina A. Tsvetikova · Elena A. Morozova · Vsevolod V. Komissarov · Yaroslav V. Tkachev · Vladimir P. Timofeev · Tatyana V. Demidkina · Nicolai G. Faleev

Received: 26 August 2010 / Accepted: 28 October 2010 / Published online: 23 November 2010

Abstract A comparative study of the kinetics and stereospecificity of isotopic exchange of the *pro*-2R- and *pro*-2S protons of glycine in $^{2}H_2O$ under the action of tyrosine phenol-lyase (TPL), tryptophan indole-lyase (TIL) and methionine γ-lyase (MGL) was undertaken. The kinetics of exchange was monitored using both ^{1}H- and ^{13}C-NMR. In the three compared lyases the stereospecificities of the main reactions with natural substrates dictate orthogonal orientation of the *pro*-2R proton of glycine with respect to the cofactor pyridoxal 5′-phosphate (PLP) plane. Consequently, according to Dunathan's postulate with all the three enzymes *pro*-2R proton should exchange faster than does the *pro*-2S one. In fact the found ratios of 2R:2S reactivities are 1:20 for TPL, 108:1 for TIL, and 1,440:1 for MGL. Thus, TPL displays an unprecedented inversion of stereospecificity. A probable mechanism of the observed phenomenon is suggested, which is based on the X-ray data for the quinonoid intermediate, formed in the reaction of TPL with L-alanine. The mechanism implies different conformational changes in the active site upon binding of glycine and alanine. These changes can lead to relative stabilization of either the neutral amino group, accepting the α-proton, or the respective ammonium group, which is formed after the proton abstraction.

Keywords α-proton exchange · Glycine · Stereospecificity · Methionine γ-lyase · Tyrosine phenol-lyase · Tryptophan indole-lyase · Pyridoxal 5′-phosphate

V. V. Koulikova · L. N. Zakomirdina · E. A. Morozova · V. V. Komissarov · Y. V. Tkachev · V. P. Timofeev · T. V. Demidkina (✉)
Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov Street, Moscow 119991, Russian Federation
e-mail: tvd@eimb.ru

O. I. Gogoleva · M. A. Tsvetikova · N. G. Faleev (✉)
Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, 28 Vavilov Street, Moscow 119991, Russian Federation
e-mail: ngfal@ineos.ac.ru

Introduction

The stereochemical enzymic mechanisms generally result from the complex reciprocal effects between the enzymes and chiral substrates in the active sites. Thus, in order to reveal the stereochemical tendencies inherent in the enzymes themselves, it is useful to examine the interactions of the enzymes with achiral substrates whose steric demands are minimal. For pyridoxal 5′-phosphate (PLP)-dependent enzymes the stereospecificities with respect to the enantiotopic α-protons of glycine seem to be an almost ideal subject for studies of this kind. According to Dunathan's postulate (Dunathan 1966) it is reasonable to expect that the isotopic exchange of these protons, catalyzed by PLP-dependent enzymes in $^{2}H_2O$, should be highly stereoselective, the configuration of the preferentially exchanging proton being determined by the stereochemistry of the main reaction, catalyzed by a given enzyme with its natural substrate. This notion was substantiated by the results of Jordan and Akhtar (1970), Akhtar et al. (1975), Shostak and Schirch (1988), and Tanaka et al. (1985), where the exchange of only one of the two enantiotopic protons was observed. More recently Malthouse et al. (1991) have elaborated an elegant experimental technique for monitoring the exchange process, based on the use of NMR ^{13}C-spectroscopy. In the course of the systematic

investigation of the isotopic exchange it was shown (Malthouse 2003) that in fact both glycine protons are exchanged although at different rates, and catalytic preferences of the exchange of the *pro*-(R) and *pro*-(S)- protons were estimated for some PLP-dependent enzymes including serine hydroxymethyltransferase (Fitzpatrick and Malthouse 1998; Malthouse 2003), tryptophan synthase (Milne and Malthouse 1995; Malthouse 2003), and catalytic antibody 15A9 (Nahon et al. 1998; Malthouse 2003).

Tyrosine phenol-lyase (TPL) (EC 4.1.99.2) (TPL) and tryptophanase (tryptophan indole-lyase) (TIL) (EC 4.1.99.1) are PLP-dependent α,β-eliminating lyases that catalyze, respectively, the decomposition of L-tyrosine leading to formation of phenol and ammonium pyruvate, and analogous reaction of L-tryptophan, affording indole and ammonium pyruvate. Because of the similarity of the three-dimensional structures of TPL (Antson et al. 1993) and TIL (Isupov et al. 1998) the mechanisms of the two enzymes are generally considered to be quite similar, and conclusions concerning catalytic particularities of one enzyme often are drawn by analogy with the other (Phillips et al. 2003). The isotopic exchange of glycine protons with these enzymes was not studied. Meanwhile this question is of considerable interest, because the two enzymes are significantly different in their ability to catalyze the racemization of alanine. TPL is able to catalyze the racemization albeit at comparatively low rate (Kumagai et al. 1970a), on the other hand no racemization reactions were ever reported for TIL.

Methionine γ-lyase (MGL) (EC 4.4.1.11) catalyzes α,γ-elimination of L-methionine to produce methylmercaptan and ammonium α-ketobutyrate (Inoue et al. 2000). Tanaka et al. (1985) have shown that MGL catalyzes the exchange of *pro*-(R)-proton of glycine. However the principal possibility of the exchange of *pro*-(S)-proton at much slower rate was not considered.

In the present work a comparative study of the kinetics and stereospecificity of the isotopic exchange of the enantiotopic protons of glycine in ^2H_2O under the action of TPL, TIL, and MGL was undertaken. We have found that in all cases both protons are exchanged, although at different rates. For TIL and MGL the observed stereospecificities agree with Dunathan's postulate, while in the case of TPL an unprecedented inversion of the stereospecificity of the isotopic exchange was found.

Materials and methods

Materials

[2-^{13}C]-glycine (99 atom %) and 2,2-[^{2}H]-glycine (98 atom %) were obtained from Aldrich Chemical Co., all other chemicals were purchased from Sigma.

Preparation and assays of the enzymes

TPL from *Citrobacter freundii*, TIL from *Proteus vulgaris*, and MGL from *Citrobacter freundii* were prepared as described previously (Chen et al. 1995; Zakomirdina et al. 2002; Manukhov et al. 2005).

Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. The concentrations of homogeneous TPL and TIL were determined from the absorbencies at 278 nm, the extinction coefficients ($\varepsilon^{1\%}_{278}$) being 8.37 for TPL (Kumagai et al. 1970b), and 9.19 for TIL (Phillips and Gollnick 1989).

The activities of TPL and TIL were determined by monitoring the rate of disappearance of S–*o*-nitrophenyl-L-cysteine (SOPC) under the action of respective enzymes. The reactions were conducted at 0.6 mM concentration of SOPC in 0.1 M potassium phosphate buffer solution, pH 7.8, containing 0.1 mM PLP, at 30°C by following the decrease in absorbance at 370 nm ($\Delta\varepsilon = -1.86 \times 10^3\ M^{-1}\ cm^{-1}$). One unit of activity was determined as the amount of enzyme catalyzing the transformation of 1 μmol of SOPC per minute. The specific activities were 10 units/mg for TPL and 45 units/mg for TIL.

The activity of MGL was determined by measuring of the rate of the reaction with S-ethyl-L-cysteine in the same buffer solution at 2.5 mM concentration of S-ethyl-L-cysteine at 30°C, using a coupled assay with NADH and lactate dehydrogenase, as described earlier for TIL (Morino and Snell 1970). The specific activity of MGL was 10 units/mg.

Preparation of stereospecifically labeled monodeuterated glycines

(R)-[^{2}H]-glycine was prepared using reaction of MGL with glycine in ^2H_2O. The reaction mixture containing 45 mg of glycine and 30 units of MGL in the presence of 0.1 mM PLP, in 1.5 ml of 50 mM potassium phosphate buffer in ^2H_2O (p^{2}H 7.6), was incubated for 5 h at 30°C. The enzyme was inactivated by heating for 5 min at 90°C and separated by centrifugation. The solvent was evaporated under vacuum, and the residue was dried at room temperature in vacuum over P_2O_5.

(S)-[^{2}H]-glycine was prepared using reaction of MGL with 2,2-[^{2}H]$_2$-glycine in water. The reaction mixture containing 62 mg of 2,2-[^{2}H]$_2$-glycine glycine and 30 units of MGL in 2 ml of 50 mM potassium phosphate buffer (pH 8.0) in the presence of 0.1 mM PLP was incubated for 5 h at 30° C. The reaction mixture was treated then as described above.

Synthesis and analysis of L-phenylalanylglycines

For conversion of glycine samples to L-phenylalanylglycine the reaction of glycine with Boc-L-Phe-ONp (dioxane,

KOH, 90°, 30°min) was used. The removal of the protecting group was carried out in trifluroacetic acid at room temperature. The dry product was then dissolved in water and extracted several times by ether to remove *p*-nitrophenol. The dipeptide product was purified by TLC on KIESELGEL 60 F_{254} using n-butanol: acetic acid: water (9:1:2.5) mixture as eluent. Synthesized L-phenylalanyl-glycines were dissolved in 0.5 ml of ^2H_2O, contained 50 mM potassium phosphate buffer, p^{2}H 7.0.

Exchange reactions

Lyophilized enzymes were dissolved in 99.8 atom % ^2H_2O containing 0.1 mM PLP. The exchange reaction was initiated by adding enzyme solution to 0.5 ml of 50 mM potassium phosphate buffer, p^{2}H 8.0, containing glycine samples at 30°C. The same procedure was used for exchange reactions with monodeuterated glycines. No loss of enzyme activity was detected during experiments.

NMR spectra

^{1}H-NMR was used to follow the exchange of the α-protons of monodeuterated glycines. ^{13}C-NMR was used to follow the exchange of the α-protons of [2-^{13}C]glycine. The sample volume was 0.5 ml in sample tubes 5 mm in diameter. Spectra were obtained at 100.618 MHz for ^{13}C nuclei (400.13 MHz for ^{1}H nuclei). Typical spectral conditions for ^{13}C nuclei were: 32,000 time-domain data points; 1.606 s acquisition time; spectral width 100 ppm.; 1 Hz exponential weighting factor; a pulse width of 12 μs was used in all experiments. For ^{1}H nuclei spectral width 25 ppm and pulse width of 3 μs were used. All spectra presented were obtained without broadband proton decoupling.

All NMR spectra were recorded using a Bruker AMXIII-400 spectrometer.

Results

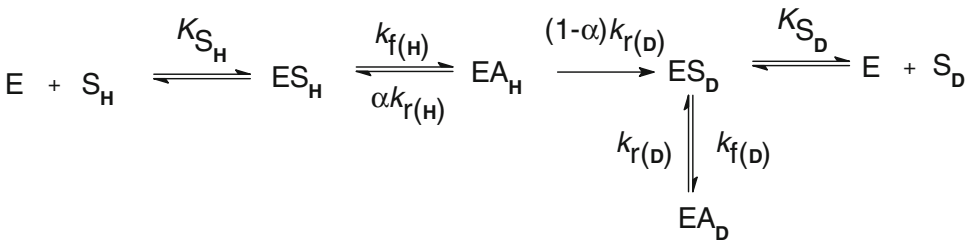
We have established that under the action of TPL, TIL and MGL on [^{13}C]-glycine in ^2H_2O its protons are exchanged for deuterons. The changes in NMR ^{13}C spectra as the reaction proceeds (data not shown), are qualitatively analogous to the data described by Malthouse et al. (1991) and Nibeilliu and Malthouse (2004), and show that as a result of the first, fast, reaction one of the protons is exchanged, leading to formation of a monodeuterated glycine. The residual proton exchanges much slower, and dideuterated glycine finally forms. To assign the absolute configurations of the monodeuterated products which are formed in the fast reactions we carried out the exchange of the ordinary glycine in ^2H_2O under the action of a given enzyme, stopped the process when the fast stage was completed, and then transformed the exchanged product into a dipeptide, L-phenylalanyl-[^{2}H]-glycine. According to (Kainosho et al. 1975) in ^{1}H NMR spectra of such dipeptides the chemical shifts of the signals of the diastereotopic protons of methylene group of glycine fragments are considerably different. In good agreement with these findings in our experiments in the case of L-PheGly, prepared from the ordinary glycine, these signals form a well defined AB-system. On the other hand, in the dipeptides, prepared from monodeuterated chiral glycines, the respective signals appear as well distinguishable singlets, located at 3.6 ppm in the case of L-Phe-(S)-[^{2}H]-Gly, and at 3.4 ppm in the case of L-Phe-(R)-[^{2}H]-Gly (data not shown). The obtained results clearly demonstrate that *pro*-(S)-proton is exchanged in fast reaction with TPL, and *pro*-(R)-one under the action of the two other enzymes.

The most of all difference in exchange rates of the two enantiotopic glycine protons was observed in the case of MGL, and this enzyme was further used for preparation of pure enantiomers of 2-[^{2}H]-glycine. To obtain (R)- 2-[^{2}H]-glycine we used the fast reaction catalyzed by MGL with normal glycine in ^2H_2O, while the same reaction with 2,2-[^{2}H]$_2$-glycine in normal water afforded the respective (S)-enantiomer. The enantiomers thus prepared were used in kinetic experiments to determine the rates of isotopic exchange of *pro*-(R)- and *pro*-(S)-protons under the action of the considered enzymes.

The overall process of isotopic exchange in amino acids may be described by the kinetic scheme 1, where the principal stages are the deprotonation of external aldimine and reprotonation of quinonoid intermediate.

The attainable degree of the exchange is determined by the isotope purity of ^2H_2O; the latter being high, the whole

Scheme 1 The principal kinetic scheme of the enzymatic isotopic exchange



reaction may be considered to be irreversible. In the frames of the suggested scheme, the whole process is irreversible because the deuteration of quinonoid EA_H, leading to aldimine ES_D, is irreversible. This implies that as a result of this stage, the α-proton, originally present in substrate S_H, is irretrievably lost. When this is considered, the quinonoids, EA_H and EA_D, are non-identical, because for the former the protonation (internal return), leading to regeneration of the initial non-deuterated substrate is still possible, while the latter can be only deuterated. Therefore, quinonoid EA_D is aside from the reaction pathway responsible for the isotopic exchange.

The extent of the internal return of the proton is determined by a coefficient α, which is associated with the rates of the isotope exchange between the enzyme functional group having abstracted the α-proton, and existing as a conjugated acid, and surrounding groups, capable of isotope exchange, and solvent molecules present in the active site. The values of K_m and k_{cat} for the isotope exchange reaction may be described by Eqs. 1 and 2.

$$K_m = \frac{K_{SH}[\alpha k_{r(H)} + (1-\alpha)k_{r(D)}]}{k_{f(H)} + \alpha k_{r(H)} + (1-\alpha)k_{r(D)}} \quad (1)$$

$$k_{cat} = \frac{(1-\alpha)k_{r(D)}k_{f(H)}}{k_{f(H)} + \alpha k_{r(H)} + (1-\alpha)k_{r(D)}} \quad (2)$$

The suggested mechanism implies also that isotope exchange reaction should be inhibited by the deuterated product. The respective inhibition constant (K_p) is described by Eq. 3.

$$K_P = \frac{K_{SD}}{1 + \frac{k_{f(D)}}{k_{r(D)}}} \quad (3)$$

In reactions of the considered enzymes with glycine no formation of any noticeable amounts of quinonoid intermediates was observed. We may suppose, consequently, that reprotonation rates strongly prevail over the rates of proton abstraction ($k_r \gg k_f$), and as a result, the life times of the quinonoid intermediates formed are very small. In this case:

$$k_{cat} = \frac{(1-\alpha)k_{r(D)}k_{f(H)}}{\alpha k_{r(H)} + (1-\alpha)k_{r(D)}} \quad (4)$$

and

$$K_m = K_p = K_{SD}. \quad (5)$$

For enzymatic reactions, where inhibition by product is observed, the dependence of product concentration on time may be described by the Foster–Niemann equation (Foster and Niemann 1953):

$$[P]\left(1 - \frac{K_m}{K_p}\right) = k_{cat}[E]_0 t - K_m\left(1 + \frac{[S]_0}{K_p}\right)\ln\frac{[S]_0}{[S]_0 - [P]} \quad (6)$$

Under the reaction conditions ($K_m = K_p$):

$$\ln\frac{[S]_0}{[S]_0 - [P]} = \frac{k_{cat}[E]_0 t}{K_m\left(1 + \frac{[S]_0}{K_p}\right)} \quad (7)$$

In all considered cases we, in fact, did observe the direct proportionality between the parameter $\ln([S]_0/([S]_0\text{-}[P]))$ and reaction time. Typical examples are presented in Figs. 1a, b, 2a, b and 3a, b. Having accepted that $K_m = K_p$ values are equal to inhibition constants (K_i) for glycine in reactions of respective enzymes with their natural substrates, we calculated the k_{cat} values for the isotopic exchange reactions catalyzed by the considered lyases from the slopes of the respective straight lines described by Eq. 7. The results are compared in Table 1 with analogous known data for some other PLP-dependent enzymes.

If the internal return of the proton does indeed play an important role in the considered reactions of isotopic exchange, the observed k_{cat} values for the exchange may be considerably lower than k_f values which characterize the rates of direct proton abstraction in the same reactions. When the values of coefficient α, and that of the isotope effect (IE) at the stage of reprotonation:

$$IE = \frac{k_{r(H)}}{k_{r(D)}} \quad (8)$$

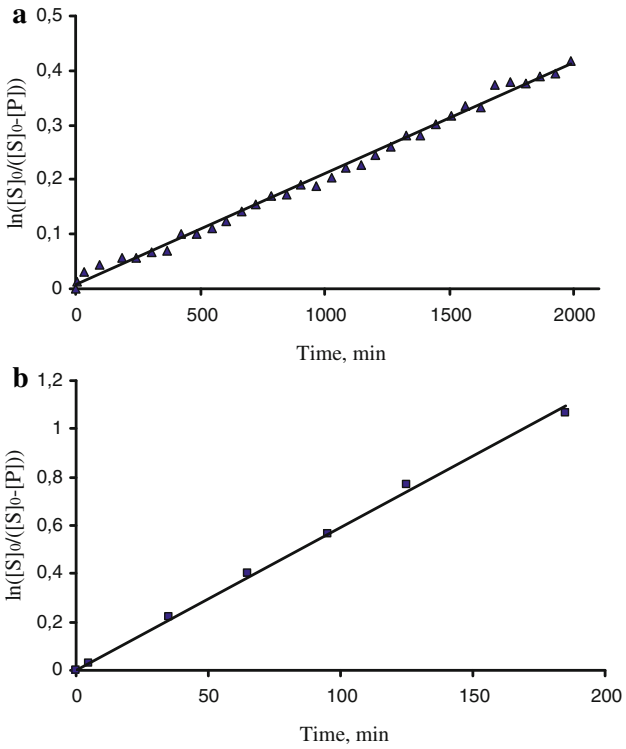


Fig. 1 **a** Isotopic exchange of (S)-2-[^{2}H]-glycine under the action of TPL. $[E]_0 = 0.171$ mM; $[S]_0 = 420$ mM. **b** Isotopic exchange of (R)-2-[^{2}H]-glycine under the action of TPL. $[E]_0 = 0.05$ mM; $[S]_0 = 400$ mM

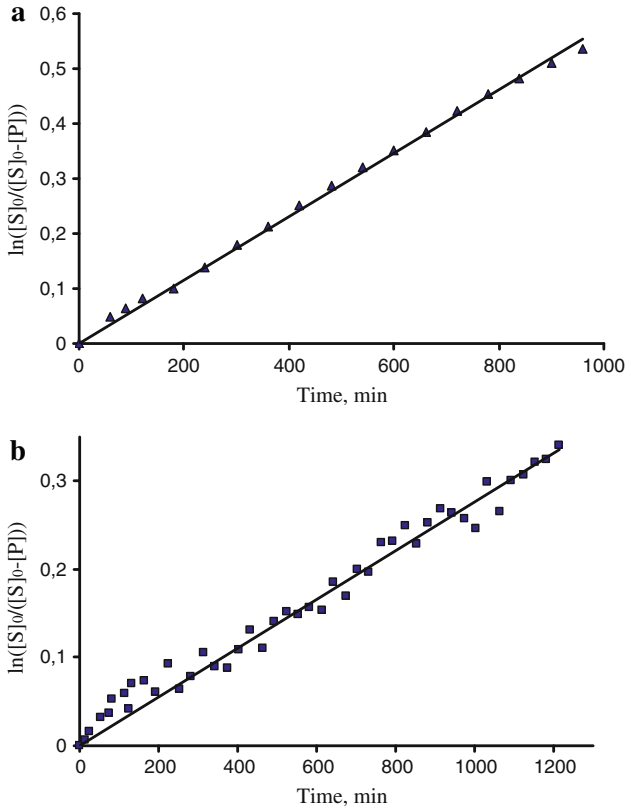


Fig. 2 **a** Isotopic exchange of (S)-2-[^{2}H]-glycine under the action of TIL. $[E]_0 = 0.06$ mM; $[S]_0 = 381$ mM. **b** Isotopic exchange of (R)-2-[^{2}H]-glycine under the action of TIL $[E]_0 = 0.360$ mM; $[S]_0 = 46$ mM

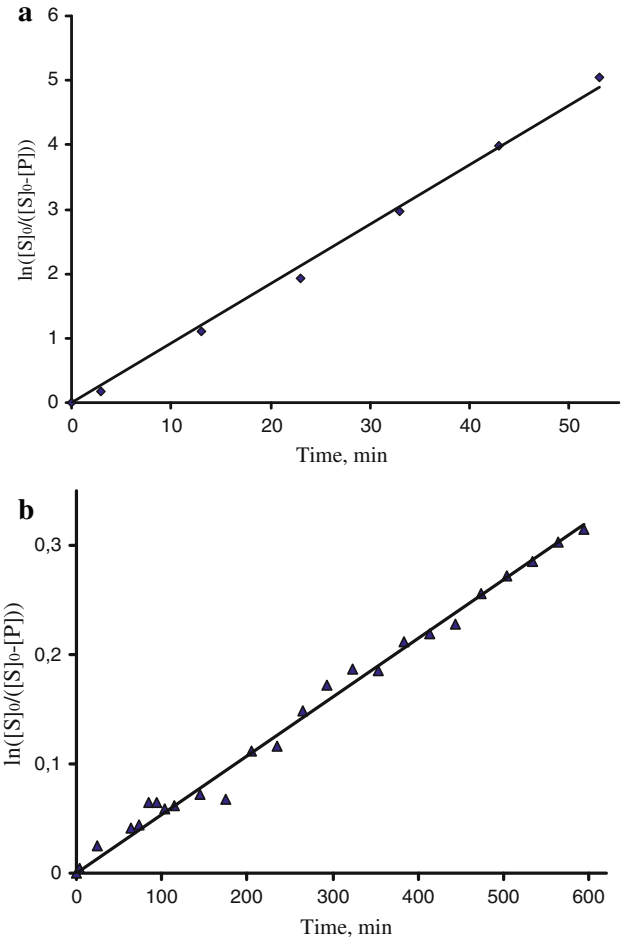


Fig. 3 **a** Isotopic exchange of (S)-2-[^{2}H]-glycine under the action of MGL $[E]_0 = 0.016$ mM; $[S]_0 = 210$ mM. **b** Isotopic exchange of (R)-2-[^{2}H]-glycine under the action of MGL $[E]_0 = 0.153$ mM; $[S]_0 = 200$ mM

are known, the k_f may be calculated from the respective k_{cat} value in accordance with equation:

$$k_{cat} = \frac{(1-\alpha)k_f}{1+\alpha(IE-1)} \quad (9)$$

which may be derived from Eqs. 4 and 8. Earlier (Faleev et al. 2004) we have studied the reaction of isotopic exchange of L-phenylalanine in ^2H_2O, catalyzed by TPL, at the level of separate elementary stages. In this enzyme the abstraction of the α-proton most likely is effected by ε-amino group of the Lys-257 residue. We have found [26] that the value of coefficient α for the studied exchange reaction is close to 0.33, which corresponds to a statistical factor for $–ND_2H^+$ group with non-restricted rotation around the C–N bond. The value of kinetic isotope effect for the reprotonation stage was found to be equal to 5.8. We accepted the same values of α and IE for the isotopic exchange reaction of glycine and calculated the value of k_f, using Eq. 9. This value is presented in Table 2, where the known kinetic parameters for the reactions of quinonoid formation under the action of TPL on L- and D-enantiomers of alanine, determined by Chen and Phillips (1993), are also given.

Discussion

In the three compared enzymes the abstraction of the α-proton from the initially formed external aldimine is a principal stage beginning the multistage transformation of the substrate (Phillips et al. 2003; Inoue et al. 2000). When the enzyme interacts with substrate analogues incapable of further chemical steps, and the reaction is conducted in ^2H_2O, the isotopic exchange of α-proton of the used analogue is observed, which is insured by the reversible formation of the quinonoid intermediate. According to the known X-ray data, the role of the catalytic base, responsible for the labilization of α-proton in amino acid substrates and their analogues in the active sites of PLP-dependent enzymes, belongs to the lysine residue which in the holoenzyme structure forms an internal aldimine bond with the cofactor. For the studied enzymes this role is played by Lys-257 in TPL, Lys-266 in TIL, and Lys-210 in MGL.

Table 1 Kinetic parameters for the isotopic exchange of enantiotopic protons of glycine under the action of some PLP-dependent enzymes

Enzyme	Exchange of "right" proton		Exchange of "wrong" proton	
	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
Serine transhydroxymethylase		4.0[a]		5.4×10^{-4}[a]
		pro-(S)		*pro*-(R)
Tryptophan synthase	36–149[b]	2.9–3.2[b]	41–175[b]	1.1 to 1.3×10^{-2}[b]
		pro-(R)		*pro*-(S)
MGL	48.5[c]	20.2	48.5	1.4×10^{-3}
		pro-(R)		*pro*-(S)
TIL	45.8[c]	6.3×10^{-2}	45.8	5.8×10^{-4}
		pro-(R)		
TPL	85[c]	4.3×10^{-2}	85	0.9
		pro-(R)		*pro*-(S)

[a] Data of Malthouse et al. (1991)

[b] Data of Nibeilliu and Malthouse (2004)

[c] K_i values for glycine in reactions with respective enzymes. The inhibitory effect of glycine on MGL was determined using L-methionine as substrate, as described by Manukhov et al. (2005). The inhibitory effects of glycine on TPL and TIL were determined using L-tyrosine and L-tryptophan as substrates, respectively, as described for TIL (Morino and Snell 1970)

Table 2 Kinetic parameters for reversible quinonoid formation in the reactions of TPL with glycine and alanine

Substrate	K_S (mM)	k_f (s^{-1})	k_r (s^{-1})
(S)-2-[^{2}H]-glycine	85[a]	0.16	too fast
(S)-alanine	80[b]	1.81[b]	0.37[b]
(R)-2-[^{2}H]-glycine	85[a]	$\geq$0.9	too fast
(R)-alanine	90[b]	0.75[b]	0.29[b]

[a] K_i determined for inhibitor glycine in the reaction with L-tyrosine

[b] Data of Chen and Phillips (1993)

In Table 1 the obtained results, characterizing the kinetics and stereoselectivity of the isotopic exchange of the enantiotopic protons of glycine under the action of the three lyases, are compared with the analogous data for other PLP-dependent enzymes.

In accordance with Dunathan's postulate, and taking into account the stereochemical features of the main reactions with natural substrates, the preferential exchange of the *pro*-(R)- proton of glycine should be expected for all the three enzymes, which is really observed in the cases of TIL and MGL. At the same time the inversion of the normal stereoselectivity of the isotopic exchange takes place for TPL, and (S)-monodeuterated glycine is formed as a product of the fast exchange reaction. Bearing in mind the known ability of TPL to catalyze racemization of alanine, it might be proposed that in the case of glycine the isotopic exchange proceeds with the inversion of configuration of α-carbon atom. According to this mechanism glycine is bound only in the "right" conformation and, consequently, *pro*-(R) proton is initially abstracted both in the case of nondeuterated and that of monodeuterated (S)-glycine. In this case the rates of sequential exchange of the two protons of glycine should not be much different because in both cases the proton is abstracted in the rate-limiting step, and the effect of the neighboring deuterium atom on the rates of deprotonation and subsequent deuteration should be small. However, in fact the rate of monodeuterated (S)-glycine formation is more than 20 times more than the rate of exchange of the residual *pro*-(R)-proton. We may conclude, therefore, that both exchange reactions proceed with the retention of α-carbon atom configuration, the *pro*-(S)-proton being exchanged in the course of the fast reaction, and the *pro*-(R)-proton during the slow one. Most likely, the exchange of the "right" *pro*-(R)-proton is effected under the action of Lys-257 residue, while for the exchange of the other proton participation of a different catalytic group is necessary. X-ray data for the TPL complexed with N-(5′-phosphopyridoxyl)-L-tyrosine, reported by Pletnev et al. (1997), allowed us to consider Tyr-71 residue as a possible candidate on this role. It should be noted, however, that a homologous residue of Tyr-72 is present in the active site of TIL. Taking into account the similarity in structures and mechanisms of TPL and TIL, mentioned above, we consider the normal stereoselectivity of isotopic exchange of enantiotopic protons of glycine under the action of TIL, established in the present work, as a serious argument against the participation of Tyr-71 residue of TPL in the fast exchange of the "wrong" proton.

The X-ray data (PDB 2VLF), presented by Milic et al. (2008) for the quinonoid intermediate, which is formed in

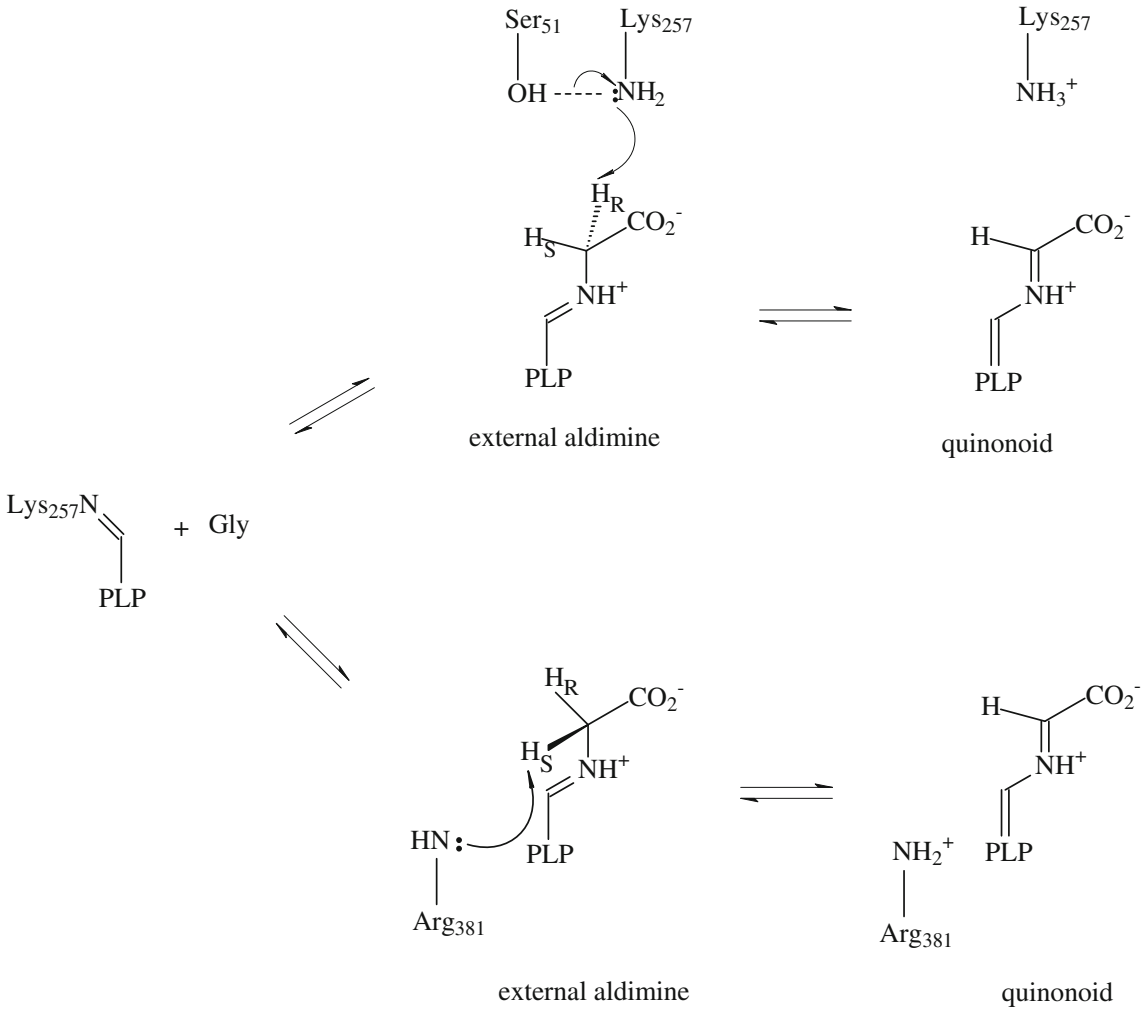
the reaction of TPL with L-alanine, show that two catalytic basic groups are located at two opposite sides of the quinonoid plane. The ε-amino group of Lys257 is placed at the re-side of the quinonoid, and Arg-381 is located at the opposite side. The latter may act either directly, or through a water molecule(s) (Wat2 and Wat3) which are found in a close proximity. In external aldimine structures, formed in the active site upon binding of the different enantiomers of alanine the α-protons of the amino acid moiety should be oriented orthogonally with respect to the PLP plane, being directed towards one or the other catalytic base. The respective quinonoid intermediates, formed from L- or D-alanine should be chemically quite identical, whereas their environments should differ in the position of the proton, abstracted from the α-position of the bound enantiomer. It seems probable that the limiting stage of the slow racemization of alanine is the transfer of the proton between the basic groups through a network of hydrogen bonds. Such process brings about a redistribution of charges in the active site, and may be accompanied by considerable conformational changes.

We may suppose, by analogy, that the external aldimine, formed upon binding of TPL with glycine, in principle, may exist in two conformations which differ in orientation of the enantiotopic protons with respect to the cofactor plane. In this case the isotopic exchange of the "right" *pro*-(R)-proton will proceed under the action of Lys-257, as a catalytic base, whereas Arg-381, and, possibly, a molecule of water should participate in the exchange of the *pro*-(S)-proton. It seems logical to suppose that in the first case the respective quinonoid intermediate should exist in a contact ion pair with the protonated amino group of Lys-257, while in the second one a solvent separated ion pair with the guanidinium group of Arg-381 should be formed. Evidently, for the solvent separated pair the exchange processes leading to washing out of the isotopic label should be more intensive, than for the contact ion pair, and these factors should assist the observed inversion of stereoselectivity of the isotopic exchange in the case of TPL. We must bear in mind, however, that upon binding of alanine enantiomers with TPL quinonoid intermediates are formed in big amounts, while no considerable formation of such intermediates was ever observed for the analogous reaction with glycine. We believe that understanding of origins of this inconsistency is essential for elucidation of the enzymatic mechanisms of both isotopic exchange of the protons of glycine and racemization of alanine.

In the general case the formation of a quinonoid intermediate implies the transfer of the α-proton from the C_α-atom of the external aldimine to a certain basic residue. The binding of various amino acids in the active site may be accompanied by conformational changes which depend on the structure of the side group. These changes may alter the mutual orientation of α-proton in the external aldimine and the catalytic base, accepting this proton, leading to retardation or acceleration of deprotonation/reprotonation processes. At the same time, if the inherent C–H acidity of the external aldimine and the basicity of the acceptor of the proton remain without considerable changes, the equilibrium ratio of the quinonoid intermediate and external aldimine also should not change much. According to a quantitative estimation presented by Nibeilliu and Malthouse (2004), C–H acidity of the external aldimine formed upon binding of glycine is not less, but even somewhat more than that of the analogous structure with alanine. Thus, the inconsistency observed for the reactions of TPL with glycine and enantiomers of alanine should be due to other factors. In this connection we paid attention to amino acid residues which are located in the active site of TPL near Lys-257, and may seriously affect the resulting basicity of the latter.

In the apoenzyme of TPL Lys-257 is in a close proximity to the side chain of Ser-51 (Milic et al. 2006). In the apoenzyme complex with N-(5′-phosphopyridoxyl)-L-tyrosine, which may be considered as a model of external aldimine structure, the amino group of Lys-257 also is at a hydrogen bond distance from Ser-51 residue. Formation of a hydrogen bond in this case implies the participation of the lone pair of Lys-257 and the hydroxylic proton of Ser-51, which should result in a decrease of the effective basicity of the lysine amino group. It seems probable that the decrease in the exchange rate of the "right" proton of glycine in the reaction with TPL (see Table 1) as compared to other enzymes is a result of this interaction. On the other hand in the quinonoid complex of TPL with L-alanine (Milic et al. 2008) the α-amino group of Lys-257, which should be protonated**,** forms hydrogen bonds with the hydroxylic groups of Ser-51 and Ser-254 residues, and also with a negatively charged oxygen of the phosphate group of PLP. In these bonds the protons of the ammonium group interact with lone pairs of respective oxygens which should result in a strong stabilization of the ammonium group, and consequently, the equilibrium, associated with the proton transfer, should be shifted to protonated lysine amino group and quinonoid intermediate, leading to the increase of the equilibrium content of the latter.

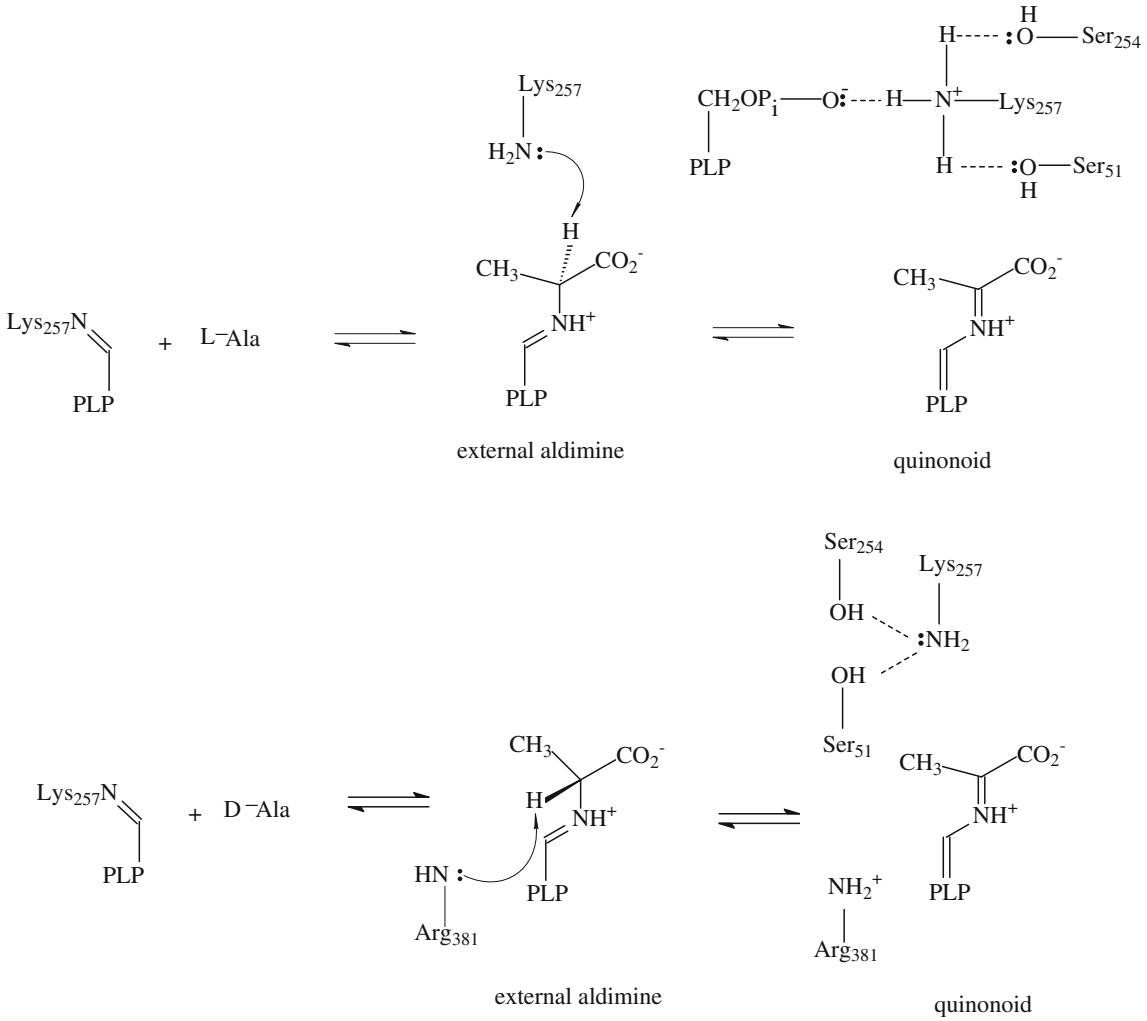
The absence of any noticeable amounts of quinonoid intermediate in the case of TPL reaction with glycine may be interpreted as evidence that the stabilizing interactions noted above are much weakened, or even absent at all. In view of these results we may conclude that conformational changes, associated with the binding of TPL with glycine or alanine, are characterized by subtle differences leading to following results. Upon binding with glycine (see Scheme 2) a hydrogen bond is formed between the neutral amino group of Lys-257 and Ser-51 residue, and the



Scheme 2 The proposed mechanism of the reversible quinonoid formation in the reaction of TPL with glycine

basicity of the amino group is decreased. The subsequent abstraction of the "right" α-proton is not accompanied by any considerable stabilization of the ammonium group being formed. On the other hand, upon binding of TPL with L-alanine (Scheme 3) the hydrogen bond between Lys-257 and Ser-51 in the external aldimine is not formed, or is considerably weakened, thus the effective basicity of the amino group increases and subsequent abstraction of α-proton is accelerated. During the abstraction of the proton, or immediately after it, conformational changes take place to the effect that positively charged ammonium group of Lys-257 becomes close enough to Ser-51 and Ser-254 residues, and to an oxygen atom of cofactor phosphate group, and thus effective stabilization of the ammonium group is gained. This results in a strong retardation of reprotonation of the quinonoid intermediate, and its equilibrium content is much increased. The data, presented in Table 2, and characterizing the abstraction of *pro*-(R)-proton of glycine and α-proton of (S)-alanine, agree with the suggested scheme. It is noteworthy that K_S values, for the formation of external aldimine in reactions with glycine and (S)-alanine are very close. Meanwhile, according to Faleev et al. (1988), the efficiency of binding of amino acids in the active site should increase as a result of the increase in hydrophobicity of the side group on passing from glycine to alanine. It is logical to suppose that in the case of L-alanine the gain in energy at the stage of external aldimine formation is spent on the breaking of the hydrogen bond between Lys-257 and Ser-51, and the subsequent conformational change, making possible the subsequent formation of the quinonoid intermediate and stabilization of the ammonium group of Lys 257, and leading, after all, to the increase of the resulting effective affinity in the case of L-alanine.

It is evident that the rate of abstraction of the "wrong" *pro*-(S)-proton of glycine is similar to the rate of α-proton abstraction for D-enantiomer of alanine (see Table 2), which shows that upon binding of glycine or D-alanine the



Scheme 3 The proposed mechanism of the reversible quinonoid formation in the reactions of TPL with L- and D-alanine

environment of Arg-381 residue does not change much, and the abstraction of the proton effected by Arg-381 in contrast to Lys-257, occurs in the two compared reactions under analogous conditions (Schemes 1, 2). The shift of the equilibrium towards the formation of quinonoid intermediate in the reaction with D-alanine, probably, is effected by conformational changes which proceed immediately after the abstraction of the proton, and lead to appearance or enhancement of definite stabilizing interactions. If these conformational changes are similar to that occurring upon binding of L-enantiomer, we may suppose that bringing of neutral Lys-257 in close proximity to Ser-51 and Ser-254 may contribute into the total stabilizing effect as a result of formation of hydrogen bonds between the basic amino group of lysine and protons of the serine residues (Scheme 3).

It is necessary to note, however, that because of the absence of noticeable amounts of quinonoid intermediate in TPL reaction with glycine even the mere fact of its formation may be called in question. Earlier (Faleev et al. 2004) we suggested a concerted mechanism of the isotopic exchange for TPL reaction with L-methionine. This mechanism implies the formation of six-membered transition state, involving a molecule of water. If an analogous mechanism is operative in the case of TPL reaction with glycine, the faster exchange of the *pro*-(S)-proton may reflect the greater accessibility of the respective external aldimine conformation for molecules of water.

Thus, the inversion of stereoselectivity of the isotopic exchange of the two enantiotopic protons of glycine in the reaction with TPL is ensured by fulfillment of the two conditions:

1. Upon binding of glycine two conformations of the external aldimine may be formed which ensure the necessary orthogonal orientation of the two enantiotopic protons at different sides of the cofactor PLP plane.
2. The presence of a catalytic base (Arg-381), capable to effect the labilization of the "wrong" proton in corresponding conformation.

It seems likely that condition 1 may be fulfilled for other PLP-dependent enzymes too, which makes the exchange of the "wrong" proton possible in principle. Relatively low rates of such exchange may be due to the fact that condition 2 is not fulfilled, and consequently, this condition becomes the main factor controlling the stereoselectivity of the isotopic exchange. The slow exchange of the "wrong" proton, observed in most cases, may imply participation of remote basic groups, acting through a network of hydrogen bonds. Otherwise, basic components of buffer solutions, penetrating the active site may be operative.

Acknowledgments This work was partly supported by grants from Russian Foundation for Basic Research (07-04-01138; 08-04-00117), and a grant of the President of Russian Federation for the support of leading scientific schools 64658.2010.4.

References

Akhtar M, El-Obeid HA, Jordan PM (1975) Mechanistic, inhibitory and stereochemical studies on cytoplasmic and mitochondrial serine transhydorxymethylases. Biochem J 145:9–168

Antson AA, Demidkina TV, Gollnick P, Dauter Z, Von Tersch RL, Long J, Berezhnoy SN, Phillips RS, Harutyunyan EH, Wilson KS (1993) Three-dimensional structure of tyrosine phenol-lyase. Biochemistry 32:4195–4206

Chen H, Phillips RS (1993) Binding of phenol and analogues to alanine complexes of tyrosine phenol lyase from Citrobacter freundii: implications for the mechanisms of α,β-elimination and alanine racemization. Biochemistry 32:11591–11599

Chen H, Gollnick P, Phillips RS (1995) Site-directed mutagenesis of His 343 → Ala in *Citrobacter freundii* tyrosine phenol-lyase, effects on the kinetic mechanism and rate-determining step. Eur J Biochem 229:540–549

Dunathan HC (1966) Conformation and reaction specificity in pyridoxal phosphate enzymes. Proc Natl Acad Sci 55:712–716

Faleev NG, Ruvinov SB, Demidkina TV, Myagkikh IV, MYu Gololobov, Bakhmutov VI, Belikov VM (1988) Tyrosine phenol-lyase from *Citrobacter intermedius.* Factors controlling substrate specificity. Eur J Biochem 177:395–401

Faleev NG, Demidkina TV, Tsvetikova MA, Phillips RS, Yamskov IA (2004) The mechanism of α-proton isotope exchange in amino acids catalysed by tyrosine phenol-lyase. What is the role of quinonoid intermediates? Eur J Biochem 271:4565–4571

Fitzpatrick TB, Malthouse JPG (1998) A substrate-induced change in the stereospecificity of the serine-hydroxymethyltransferase-catalysed exchange of the alfa-protons of amino acids -evidence for a second catalytic site. Eur J Biochem 252:113–117

Foster RJ, Niemann C (1953) The evaluation of the kinetic constants of enzyme catalyzed reactions. Biochemistry 39:999–1003

Inoue H, Inagaki K, Adachi N, Tamura T, Esaki N, Soda K, Tanaka H (2000) Role of tyrosine 114 of L-methionine-γ-lyase lyase from *Pseudomonas putida*. Biosci Biotechnol Biochem 64:2336–2343

Isupov MN, Antson AA, Dodson GG, Dodson EJ, Dementieva IS, Zakomirdina LN, Wilson KS, Dauter Z, Harutyunyan EH (1998) Crystal structure of tryptophanase. J Mol Biol 276:603–623

Jordan PM, Akhtar M (1970) The mechanism of action of serine transhydroxymethylase. Biochem J 116:277–286

Kainosho M, Ajisaka K, Kamisaku M, Murai A (1975) Conformational analysis of amino acids and peptides using specific isotope substitution. I. Conformation of L-phenylalanylglycine. Biochem Biophys Res Commun 64:425–432

Kumagai H, Kashima N, Yamada H (1970a) Racemization of D- or L-alanine by crystalline tyrosine phenol-lyase from *Escherichia intermedia*. Biochem Biophys Res Commun 39:796–801

Kumagai H, Yamada H, Matsiu H, Ohkishi H, Ogata K (1970b) Tyrosine phenol-lyase: I. Purification, crystallization, and properties. J Biol Chem245:1767–1777

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Malthouse JPG (2003) Stereospecificity of α-proton exchange reactions catalysed by pyridoxal-5′-phosphate-dependent enzymes. Biochim Biophys Acta 1647:138–142

Malthouse JPG, Milne JJ, Gariani LS (1991) A comparative study of the kinetics and stereochemistry of the serine hydroxymethyltransferase- and tryptophan synthase-catalysed exchange of the pro-2R and pro-2S protons of glycine. Biochem J 274:807–812

Manukhov IV, Mamaeva DV, Rastorguev SM, Faleev NG, Morozova EA, Demidkina TV, Zavilgelsky GB (2005) A gene encoding L-methionine γ-lyase is present in *Enterobacteriaceae* family genomes: Identification and characterization of *Citrobacter freundii* L-methionine gamma-lyase. J Bacteriol 187:3889–3893

Milić D, Matković-Calogović D, Demidkina TV, Kulikova VV, Sinitzina NI, Antson AA (2006) Structures of apo- and holotyrosine phenol-lyase reveal a catalytically critical closed conformation and suggest a mechanism for activation by K^+ ions. Biochemistry 45:7544–7552

Milić D, Demidkina TV, Faleev NG, Matković-Calogović D, Antson AA (2008) Insights into the catalytic mechanism of tyrosine phenol-lyase from X-ray structures of quinonoid intermediates. J Biol Chem 283:29206–29214

Milne JJ, Malthouse JPG (1995) Factors affecting the stereospecificity and catalytic efficiency of the tryptophan synthase-catalysed exchange of the pro-2R and pro-2S protons of glycine. Biochem J 311:1015–1019

Morino Y, Snell EE (1970) Tryptophanase (*Escherichia coli* B). Methods Enzymol 17A:439–446

Nahon MM, Gramatikova SI, Christen P, Fitzpatrick TB, Malthouse JPG (1998) The pyridoxal 5′-phosphate-dependent catalytic antibody 15A9: its efficiency and stereospecificity in catalysing the exchange of the alfa-protons of glycine. FEBS Lett. 427:74–78

Nibeilliu ME, Malthouse JP (2004) The stereospecificity and catalytic efficiency of the tryptophan synthase-catalysed exchange of the α-protons of amino acids. Biochem J 381:847–852

Phillips RS, Gollnick PD (1989) Evidence that cysteine 298 is in the active site of tryptophan indole-lyase. J Biol Chem 264:10627–10632

Phillips RS, Demidkina TV, Faleev NG (2003) Structure and mechanism of tryptophan indole-lyase and tyrosine phenol-lyase. Biochim Biophys Acta 1647:167–172

Pletnev SV, Antson AA, Sinitsina NI, Dauter Z, Isupov MN, Hurs EN, Faleev NG, Wilson KS, Dodson G, Demidkina TV, Harutyunyan EG (1997) Crystallographic investigation of tyrosine phenol-lyase from *Erwinia herbicola*. Kristallografia (in Russian) 42:877–888

Shostak K, Schirch V (1988) Serine hydroxymethyltransferase: mechanism of the racemization and transamination of D- and L-alanine. Biochemistry 27:8007–8014

Tanaka H, Esaki N, Soda K (1985) A versatile bacterial enzyme: L-methionine-γ-lyase. Enz Microb Technol 7:530–537

Zakomirdina LN, Kulikova VV, Gogoleva OI, Dementieva IS, Faleev NG, Demidkina TV (2002) Tryptophan indole-lyase from *Proteus vulgaris*: kinetic and spectral properties. Biochemistry (Mosc) 67:1437–1445