The effect of different amino acid side chains on the stereospecificity and catalytic efficiency of the tryptophan synthase-catalysed exchange of the α -protons of amino acids

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¹H-NMR has been used to follow the tryptophan synthase (EC 4.2.1.20) catalysed hydrogen–deuterium exchange of the α -protons of L- and D-alanine and -tryptophan. The first-order and second-order rate constants for exchange have been determined at pH 7.8 in the presence and absence of the allosteric effector, DL- α -glycerol 3-phosphate. In the presence of DL- α -glycerol 3-phosphate the stereospecificity of the tryptophan synthase-catalysed first-order exchange rates was in the order tryptophan > alanine > glycine. This increase in stereospecificity was largely due to the decrease in the magnitude of the first-order exchange rate of the slowly exchanged α -proton. A similar increase in the stereospecificity of the second-order exchange rates for alanine was also largely due to the decrease in the magnitude of the first-

order exchange rate of the slowly exchanged α -proton of D-alanine. Adding DL- α -glycerol 3-phosphate produced an increase in the stereospecificity of the second-order exchange rate observed with alanine but no significant change in the stereospecificity of the first-order exchange rate with tryptophan. The α -subunits are shown to increase the exchange rates of the α -protons of L-alanine and L-tryptophan. We conclude that the contribution of the R-group of an amino acid to the stereospecificity of the exchange reactions of its α -proton can be similar to or larger than that of its α -carboxylate group. Possible mechanisms that could explain the stereospecificity of these exchange reactions are discussed.

INTRODUCTION

Pyridoxal phosphate-dependent enzymes catalyse a wide range of reactions that can involve cleavage of the α -carbon bond of amino acids [1,2]. In the holoenzyme the pyridoxal phosphate cofactor forms an internal aldimine with the ϵ -amino group of an active-site lysine residue, which is then displaced when the incoming amino acid forms an external aldimine with the cofactor. It has been argued that for optimal catalytic efficiency the α -carbon bond to be cleaved should be orthogonal to the plane of the imine-cofactor π -electron system [3]. The reaction catalysed will depend on whether the amino acid R-group, α -carboxylate or α -proton is orthogonal to the plane of the imine-cofactor π -electron system. Therefore the stereospecificity of the exchange of the α -protons of L- and D- amino acids or of the pro-2R or pro-2S protons of glycine will depend on how the carboxylate and R-groups are bound relative to the plane of the imine-cofactor π -electron system [3,4]. For an exchange of α -protons we would expect the degree of stereospecificity to depend on the efficiency of binding of the amino acid R-group and α -carboxylate group.

All the reactions of tryptophan synthase (EC 4.2.1.20) result from the primary cleavage of the α -proton of L-amino acids [5] and proton transfers are thought to occur on the Si face of the cofactor ring [6,7]. Using an NMR-kinetic technique we have shown that tryptophan synthase preferentially catalyses the exchange of the pro-2R proton of glycine at a rate 290 times faster than that of the pro-2S proton of glycine [8,9]. Recently we have shown that both the α -subunits and the allosteric effector DL- α -glycerol 3-phosphate decrease the stereospecificity of tryptophan synthase [9]. DL- α -Glycerol 3-phosphate can be considered as either an analogue of D-glyceraldehyde 3-phosphate or of indol-3-ylglycerol phosphate, which binds to the indol-3-ylglycerol phosphate binding site on the α -subunit [5,10–12]. It has no effect on the catalytic properties of isolated β -subunits but

when it binds to the α -subunits of the $\alpha_2\beta_2$ enzyme complex it increases the rate of some catalytic steps while decreasing the rates of others. It has been suggested that DL- α -glycerol 3-phosphate's binding to the α -subunits of the $\alpha_2\beta_2$ enzyme complex either causes a conformational change at the active site of the β -subunits [13] or preferentially stabilizes a pre-existing $\alpha_2\beta_2$ enzyme conformation [10,13].

In the present study we have determined the tryptophan synthase-catalysed exchange rates of the α -protons of L- and D-alanine and -tryptophan in the presence and absence of DL- α -glycerol 3-phosphate. By comparing these rates with those obtained for the exchange of the α -protons of glycine under the same conditions we are able to show how the size of the R-group affects the stereospecificity of the tryptophan synthase-catalysed exchange of the α -protons of amino acids. Dunathan [3] has assumed that the binding of the carboxylate anion will be the most important factor in controlling reaction specificity of pyridoxal phosphate-dependent enzymes. However, our results show that for tryptophan synthase the R-group can make a significant contribution to the specificity of the exchange of α -protons of amino acids. We argue that our results cannot be explained by a mechanism in which the α -carboxyl groups of both L- and D-amino acids bind at the same site. Instead we argue that our results are consistent with a mechanism where the binding sites of the R-group and α -carboxylate group are exchanged with L- and D-amino acids.

MATERIALS AND METHODS

Materials

[2-¹³C]Glycine (99 at. %) and ²H₂O (99.8 at. %) were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). All other chemicals used were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

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Methods

Isolation and assay of tryptophan synthase and its α and β_2 subunits

The tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* was purified from the *Escherichia coli* strain CB149 that lacks the *trp* operon and harbours the high-copy plasmid pSTB7 containing the wild-type *trpA* and *trpB* genes from *S. typhimurium* (provided by Dr. Edith Wilson Miles) as described by Miles et al. [14]. The α -subunit was isolated from the $\alpha_2\beta_2$ enzyme complex by acid precipitation of the β -subunit at pH 4.5 [15,16]. The β_2 subunit was isolated from the $\alpha_2\beta_2$ enzyme complex by heat denaturation at 63 °C of the α -subunit at pH 7.8 [16,17].

The concentration of the functional active sites of the isolated β -subunits and the β -subunits within the $\alpha_2\beta_2$ enzyme complex were determined as described by Milne and Malthouse [9].

NMR samples

All solutions were made up in 99.8 at. % $^2\text{H}_2\text{O}$. Freeze-dried samples of the $\alpha_2\beta_2$ enzyme complex and samples of the α - and β -subunits were made up in solutions containing 99.8 at. % $^2\text{H}_2\text{O}$. For ^1H -NMR studies with L-alanine, D-alanine, L-tryptophan and D-tryptophan the enzyme-catalysed exchange reactions were initiated by adding 0.015–0.1 ml of enzyme to give a final volume of 0.5 ml. The accumulation of spectra occurred 4–7 min after addition of the enzyme. When the NMR observations were completed, the sample's temperature, pH and catalytic activity were determined. Except when otherwise stated, all samples were in 0.05 M potassium phosphate buffer. Sample temperatures were 27 ± 1 °C and no loss of enzyme activity was detected during the experiments. pH values were determined at the end of the observations as described by Malthouse et al. [8].

NMR spectra

^1H -NMR was used to follow the exchange of the α -proton of the amino acids studied. Spectra were recorded by using a Bruker WP80 wide-bore NMR spectrometer operating at 80.0 MHz for ^1H nuclei. The sample size was 0.5 ml in sample tubes 5 mm in diameter. The experimental conditions used were: 4000 time-domain data points; 2.56 s acquisition time; a 2 s relaxation delay during which the residual H^2HO signal in the 99.8 at. % $^2\text{H}_2\text{O}$ was suppressed by presaturation; spectral width 10 p.p.m.; 0.4 Hz exponential weighting factor and a 44° pulse.

Observation of the hydrogen–deuterium exchange rates of the α -protons in alanine and tryptophan

^1H -NMR was used to determine the extent of hydrogen–deuterium exchange at the α -carbons of L-tryptophan, D-tryptophan, L-alanine and D-alanine. The exchange of the rapidly exchanged α -proton of L-tryptophan was followed by the decrease in intensity of the α -CH quartet at 4.0 p.p.m. whereas the exchange of the slowly exchanged α -proton of D-tryptophan was followed by the increase in intensity of the signals at 3.3 p.p.m. resulting from the AB spectrum of the β -CH₂ protons of [2- ^2H]tryptophan [18]. The spectra of L- and D-alanine at an operating frequency of 80 MHz and at pH 7.8 consist of a quartet at 3.79 p.p.m. (1 H, q, J 7 Hz) due to the α -protons and a doublet at 1.48 p.p.m. (3 H, d, J 7 Hz) due to the β -protons. In the spectrum of [2- ^2H]alanine under the same conditions the quartet centred at 3.79 p.p.m. due to the α -protons is not present, whereas the doublet due to the β -protons is replaced by a singlet at 1.48 p.p.m. The exchange of the rapidly exchanged α -proton

of L-alanine was followed by the decrease in intensity of the doublet signals centred at 1.48 p.p.m. The exchange of the slowly exchanged α -proton of D-alanine was followed by the increase in the intensity of the singlet at 1.48 p.p.m. due to the β -CH₃ protons of [2- ^2H]alanine.

RESULTS AND DISCUSSION

Kinetic analysis of the hydrogen–deuterium exchange reactions catalysed by tryptophan synthase

Scheme 1 represents the minimal kinetic scheme for the tryptophan synthase-catalysed exchange of the α -proton of glycine, alanine and tryptophan, and eqn. (1) is the differential rate equation for hydrogen–deuterium exchange according to Scheme 1 [8]:

$$+ \frac{d[\text{SD}]}{dt} = \frac{k_a[\text{E}]_0([\text{SD}]_\infty - [\text{SD}])}{[\text{S}]_t + K + C[\text{SD}]} \quad (1)$$

The exchange of the slowly exchanged pro-2S proton of glycine and the slowly exchanged α -protons of D-alanine and D-tryptophan were followed by initial rate measurements. For such initial rate measurements the term $C[\text{SD}]$ is negligible and k_{obs} values were determined by using eqn. (2) [8].

$$+ \frac{d[\text{SD}]}{dt} = \frac{k_a[\text{E}]_0[\text{SD}]_\infty}{[\text{S}]_t + K} \quad (2)$$

The integrated form (eqn. 3) of eqn. (1) was used to analyse complete progress curves of the tryptophan synthase-catalysed hydrogen–deuterium exchange of the rapidly exchanged α -protons of L-tryptophan and L-alanine, as described by Malthouse et al. [8]:

$$\frac{1}{[\text{SD}]} \left[\ln \left(\frac{[\text{SD}]_\infty - [\text{SD}]}{[\text{SD}]_\infty} \right) \right] = -k_{\text{obs}} \left(\frac{t}{[\text{SD}]} \right) - D; \\ k_{\text{obs}} = \frac{k_a[\text{E}]_0/(1+xC)}{K/(1+xC) + [\text{S}]_t}; \quad D = \frac{C/(1+xC)}{K/(1+xC) + [\text{S}]_t}; \quad x = \frac{[\text{SD}]_\infty}{[\text{S}]_t} \quad (3)$$

Scheme 1 Minimal kinetic scheme for the tryptophan synthase-catalysed exchange of the α -proton of amino acids

E, SH and SD are the free enzyme, the non-deuterated substrate and the deuterated substrate respectively. ESH and ESD are Schiff bases formed between pyridoxal 5'-phosphate and the α -amino group of the non-deuterated and deuterated amino acids respectively. EQ is a quinonoid intermediate resulting from the loss of either a proton from ESH or a deuteron from ESD.

Table 1 Kinetic parameters for the exchange of the α -protons of [2- ^{13}C]glycine, L-alanine and L-tryptophan at pH 7.8 catalysed by the $\alpha_2\beta_2$ enzyme complex of tryptophan synthase

Amino acid	2 <i>R</i> proton				2 <i>S</i> proton			Stereospecificity	
	k_a (s^{-1})	K (M)	k_a/K ($\text{M}^{-1} \cdot \text{s}^{-1}$)	C	k_a (s^{-1})	K (M)	k_a/K ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{ar}/k_{as}	$(k_a/K)_r/(k_a/K)_s$
Gly*	3.2	0.054	59.0	< 0.10	0.011	0.041	0.27	290	220
Ala	8.6	0.273	31.6	0.31	≤ 0.033	≥ 2.0	0.0165	≥ 261	1920
Trp	1.7	≤ 0.010	≥ 170.0	0.53	0.000125	≤ 0.010	≥ 0.0125	13600	†

* Data for the pro-2*R* and pro-2*S* protons of glycine obtained by Milne and Malthouse [9].

† Not determined.

Table 2 Kinetic parameters for the exchange of the α -protons of [2- ^{13}C]glycine, L-alanine, D-alanine, L-tryptophan and D-tryptophan catalysed by the $\alpha_2\beta_2$ enzyme complex of tryptophan synthase at pH 7.8 in the presence of 50 mM DL- α -glycerol 3-phosphate

Amino acid	2 <i>R</i> proton				2 <i>S</i> proton			Stereospecificity	
	k_a (s^{-1})	K (M)	k_a/K ($\text{M}^{-1} \cdot \text{s}^{-1}$)	C	k_a (s^{-1})	K (M)	k_a/K ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{ar}/k_{as}	$(k_a/K)_r/(k_a/K)_s$
Gly*	1.8	0.024	75.0	< 0.10	0.011	0.0032	3.4	160	22
Ala	5.1	0.040	127.7	0.31	0.0048	0.271	0.0176	1060	7260
Trp	1.6	≤ 0.010	≥ 160.0	†	0.00012	≤ 0.010	≥ 0.014	13300	†

* Data for the pro-2*R* and pro-2*S* protons of glycine obtained by Milne and Malthouse [9].

† Not determined.

The effect of the size of the R-group of amino acids on the catalytic efficiency and stereospecificity of the tryptophan synthase-catalysed exchange of the α -protons of amino acids at pH 7.8

Saturation kinetics were not observed during the exchange of the slowly exchanged α -proton of D-alanine. We estimate that the apparent K_a for this reaction must be greater than or equal to 2.0 M. Using this estimate of K_a and the experimentally determined second-order rate constant we calculate that the first-order exchange rate for the slowly exchanged α -proton of D-alanine is no greater than 0.033 s^{-1} (Table 1). Therefore the stereospecificity of the tryptophan synthase-catalysed first-order exchange of the α -protons of L- and D-alanine is similar to or better than that of the equivalent exchange of the pro-2*R* and pro-2*S* protons of glycine (Table 1). The stereospecificity of the first-order exchange of the α -protons of L- and D-tryptophan is approx. 50 times that of the pro-2*R* and pro-2*S* protons of glycine (Table 1). Therefore the larger R-group of tryptophan increases the stereospecificity of the first-order exchange rate. This increase in stereospecificity is not achieved by increasing the first-order exchange rate of the rapidly exchanged α -proton of L-tryptophan; instead it is achieved by decreasing the first-order exchange rate of the slowly exchanged α -proton of D-tryptophan relative to the equivalent pro-2*S* proton of glycine (Table 1). Therefore the better binding by the larger R-group of tryptophan is used mainly to decrease the first-order exchange rate of the slowly exchanged α -proton of D-tryptophan. Similarly the larger R-group of alanine increases the stereospecificity of the second-order exchange reaction approx. 9-fold by decreasing the exchange rate of the slowly exchanged α -proton of D-alanine. Owing to the tight binding of tryptophan we were unable to determine the second-order exchange rates for the α -protons of

L- and D-tryptophan and we were therefore unable to determine the stereospecificity of the second-order exchange reactions involving these amino acids (Table 1).

The effect of the size of the R-group of amino acids on the catalytic efficiency and stereospecificity of the tryptophan synthase-catalysed exchange of the α -protons of amino acids at pH 7.8 in the presence of DL- α -glycerol 3-phosphate

Spectrophotometric studies have shown that 50 mM DL- α -glycerol 3-phosphate decreases the apparent K_a for the binding of amino acids such as glycine, histidine and tryptophan [10,13]. The apparent K_a values obtained from studying the exchange of the pro-2*R* and pro-2*S* protons of glycine were also decreased in the presence of 50 mM DL- α -glycerol 3-phosphate [9]. Likewise DL- α -glycerol 3-phosphate decreased the apparent K_a when the α -protons of L- and D-alanine were exchanged (Tables 1 and 2). We were therefore able to determine the stereospecificity of the first-order exchange rates for alanine as well as tryptophan (Table 2). The stereospecificity of the first-order exchange reactions was in the order tryptophan > alanine > glycine (Table 2). This shows that the stereospecificity of exchange increases as the size of the R-group increases and that the R-group can increase the stereospecificity of the first-order exchange reaction by as much as 80-fold (Table 2). This increase in stereospecificity as the size of the R-group increases is due mainly to a decrease in the magnitude of the first-order exchange rate for the slowly exchanged 2*R* proton (Table 2). Increasing the R-group from a H- to a CH_3 - group increases the stereospecificity of the first- and second-order exchange rates by 6.6-fold and 330-fold respectively (Table 2). Likewise the α -subunits and DL- α -glycerol 3-phosphate induce greater changes in the stereo-

Table 3 Kinetic parameters for the exchange of the pro-2R protons of [2-¹³C]glycine, L-alanine and L-tryptophan catalysed by the β -subunits of tryptophan synthase at pH 7.8

Amino acid	k_a (s ⁻¹)	K (M)	$k_a K$ (M ⁻¹ ·s ⁻¹)	$k_a(\alpha_2\beta_2)/k_a(\beta_2)$	$K(\alpha_2\beta_2)/K(\beta_2)$	$[k_a/K(\alpha_2\beta_2)]/[k_a/K(\beta_2)]$
Gly*	2.0	0.092	21.0	1.6	0.59	2.8
Ala	0.33	0.167	2.0	26.1	1.63	15.8
Trp	0.084	≤ 0.010	≥ 8.4	20.2	†	†

* Data for the pro-2R protons of glycine obtained by Milne and Malthouse [9].

† Not determined.

specificity of the second-order exchange rates of glycine than in the first-order exchange rates [9].

Adding DL- α -glycerol 3-phosphate decreased the stereospecificity of the glycine exchange reactions, but it produced an increase in the stereospecificity of the second-order exchange rate observed with alanine, and no significant change in the stereospecificity of the first-order exchange rate with tryptophan (Tables 1 and 2). In general, DL- α -glycerol 3-phosphate has larger effects on the stereospecificity of exchange reactions with glycine than with tryptophan and alanine (Tables 1 and 2).

The effect of the α -subunits on the catalytic efficiency of the tryptophan synthase-catalysed exchange of the pro-2R proton of glycine and the α -protons of L-alanine and L-tryptophan at pH 7.8

The α -subunits cause a small, approx. 50 %, decrease in the apparent binding constant for both glycine and L-alanine (Table 3). However, they cause a much larger increase in the first- and second-order exchange rates of the α -proton of L-alanine than that observed with first- and second-order exchange rates of the rapidly exchanged pro-2R proton of glycine (Table 3). The exchange rate of the α -proton of L-tryptophan was independent of the L-tryptophan concentration (10–40 mM) for both the $\alpha_2\beta_2$ enzyme complex and the β -subunits; we therefore conclude that for both the $\alpha_2\beta_2$ enzyme complex and β -subunits the apparent binding constant (K) for the exchange reaction is less than 10 mM. This is consistent with our spectrophotometric studies on *S. typhimurium* tryptophan synthase, from which we estimate that the apparent binding constant for L-tryptophan is 0.25 mM, which is similar to the values obtained with *E. coli* tryptophan synthase [10,16,19]. Therefore, although we cannot determine the effect of the α -subunits on the second-order exchange rate, we are able to show that for L-tryptophan the α -subunits cause a 20-fold increase in the first-order exchange rate, similar to that observed with L-alanine (Table 3). This result is consistent with the suggestion that the major effect of the α -subunits on the catalytic power of the β -subunits is to accelerate the rate of abstraction of the α -proton of L-serine [12,20]. It also shows that an R-group larger than that of glycine is required to maximize the effect of the α -subunits on exchange rates (Table 3).

The relative contributions of the R-group and α -carboxylate group to the stereospecificity of exchange reactions

Dunathan [3] has assumed that the binding of the carboxylate anion is the most important factor in controlling the reaction specificity of pyridoxal phosphate-dependent enzymes. We assume that with glycine it is the α -carboxylate group that determines the stereospecificity of the exchange of the α -protons.

However, our results show that increasing the size of the R-group increases the stereospecificity of both the first- and second-order exchange rates in the presence (Table 2) and absence (Table 1) of DL- α -glycerol 3-phosphate. The increases in the stereospecificity of the first-order exchange rates due to the R-group of tryptophan are smaller but are of the same magnitude as those due to the α -carboxylate group (Tables 1 and 2). However, in the presence of DL- α -glycerol 3-phosphate the increase in the stereospecificity of the second-order rate constant due to the R-group of alanine is 15 times greater than that observed with glycine under the same conditions (Table 2). Therefore our results show that for tryptophan synthase the R-group can make a contribution that is similar to or larger than that of the α -carboxylate group to the stereospecificity of α -proton exchange reactions.

Molecular basis of the stereospecificity of exchange

For first-order exchange reactions, increases in stereospecificity could arise from the binding energy being used to increase the first-order exchange rate of the rapidly exchanged 2R proton. However, our results show that this mechanism is not being used (Tables 1 and 2). The increase in stereospecificity is mainly due to the first-order exchange rate of the slowly exchanged pro-2S proton decreasing as the size of the R-group increases (Tables 1 and 2).

We shall discuss two possible mechanisms of exchange. In the first mechanism we assume that the α -carboxylate group of both D- and L-amino acids can be bound only at the same site [site A of structures (1) and (2) in Scheme 2a] and that the rapidly exchanged α -proton of an L-amino acid will be on the Si face of the cofactor ring and orthogonal to the plane of the imine-cofactor π -electron system [structure (1) in Scheme 2a]. The slowly exchanged α -proton of a D-amino acid will be [in site B of structure (2) in Scheme 2a] 109° from the position of the rapidly exchanged proton [in site C of structure (1) in Scheme 2a] and in the plane of the cofactor ring. The slow exchange rate of the α -proton of a D-amino acid would be expected to be due to its not being orthogonal to the Si face of the cofactor ring. For the α -proton of D-amino acids to be exchanged, the R-group of the D-amino acid would have to occupy the same position [site C of structures (1) and (2) in Scheme 2a] as that occupied by the α -proton of L-amino acids. For D-amino acids, increasing steric hindrance of the R-group as its size increased could explain the increase in the stereospecificity of the second-order exchange rates as the size of the R-group increases. But this mechanism would not explain the increase in the stereospecificity of the first-order exchange rates as the size of the R-group increases. A similar model in which the R-groups of both L- and D-amino acids can only bind at the same site would not explain

Scheme 2 The binding of L- and D-amino acids in two possible mechanisms

See text for details.

why the first-order exchange rates decrease as the size of the R-groups of D-amino acids increase.

In the second mechanism we assume that for both D- and L-amino acids the exchanged proton must be in the same position [in site C in structures (1) and (2) of Scheme 2b] on the Si face of the cofactor ring and orthogonal to the plane of the imine-cofactor π -electron system. We also assume that the α -carboxylate group and the R-group can exchange binding sites. When the α -carboxylate group is bound at one site [site A of structure (1) in Scheme 2b] and the R-group at another site [site B of structure (1) in Scheme 2b], the α -proton of an L-amino acid will be perpendicular to the plane of the imine-cofactor π -electron system. However, for a D-amino acid the carboxyl group and R-group must exchange binding sites [structure (2) in Scheme 2b] if the α -proton of the D-amino acid is to occupy the same position

perpendicular to the plane of the imine-cofactor π -electron system (Scheme 2b). Therefore the specificity will depend on the populations of the two productive binding modes [structures (1) and (2) in Scheme 2b] relative to the populations of their non-productive binding modes (not shown). Increases in stereospecificity as the size of the R-group increases could reflect better binding by larger R-groups in site B [structure (1) in Scheme 2b] or it could reflect their decreased binding in site A [structure (2) in Scheme 2b] owing to increasing steric hindrance as the size of the R-group increases. The observed increases in stereospecificity are mainly due to the first-order exchange rate of the slowly exchanged D-protons decreasing as the size of the R-group increases (Tables 1 and 2). It is not due to increases in exchange rate of the rapidly exchanged L-protons (Tables 1 and 2). Therefore, according to this model, the observed increases in stereospecificity must reflect decreased binding in site A [structure (2) in Scheme 2b] owing to increasing steric hindrance as the size of the R-group increases (Tables 1 and 2).

In our earlier studies [8] we favoured the first mechanism to explain our exchange rates with glycine. However, the results obtained in the present study show that this mechanism will not explain the exchange rates observed with L- and D-amino acids. Therefore we favour the second mechanism to explain our results with L- and D-amino acids. Assuming that the binding and steric hindrance due to the R-group of glycine will be negligible, we conclude that either mechanism could apply to the exchange of the α -protons of glycine.

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