The stereospecificity and catalytic efficiency of the tryptophan synthase-catalysed exchange of the α-protons of amino acids

Máire E. NiBEILLIÚ and J. Paul G. MALTHOUSE1
Centre for Synthesis and Chemical Biology, Conway Institute of Biomolecular and Biomedical Research, Department of Biochemistry, University College Dublin, Dublin 4, Ireland

13C-NMR has been used to follow the tryptophan synthase (EC 4.2.1.20) catalysed hydrogen–deuterium exchange of the pro-2R and pro-2S protons of [2-13C]glycine at pH 7.8. 1H-NMR has also been used to follow the tryptophan-synthase-catalysed hydrogen–deuterium exchange of the α-protons of a range of L- and D-amino acids at pH 7.8. The pKα values of the α-protons of these amino acids have been estimated and we have determined whether or not their exchange rates can be predicted from their pKα values. With the exception of tryptophan and norleucine, the stereospecificities of the first-order α-proton exchange reactions are independent of the size and electronenegativity of the amino acid R-group. Similar results are obtained with the second-order α-proton exchange rates, except that both L-tryptophan and L-serine have much higher stereospecificities than all the other amino acids studied.

Key words: hydrogen–deuterium exchange, NMR, α-proton, stereospecificity, tryptophan synthase.

INTRODUCTION

We have undertaken a programme of research [1,2] to quantify the catalytic efficiency and stereospecificity of the exchange of the α-protons of amino acids catalysed by serine hydroxymethyltransferase [3–5], a catalytic antibody [6], aspartate aminotransferase [7] and tryptophan synthase [5,8–11].

During catalysis by pyridoxal-phosphate-dependent enzymes, the incoming amino acid forms an external aldimine with the pyridoxal phosphate 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 [12]. It is the binding of the amino acid R-group and α-carboxylate group that should fix the appropriate α-carbon bond in position for cleavage. Therefore it is expected that the stereospecificity of the exchange of the α-protons of L- and D-amino acids or of the pro-2R and Pro-2S protons of glycine will depend on the efficiency of binding of the amino acid R-group and α-carboxylate group. Dunathan [12] has assumed that binding of the α-carboxylate anion will be the most important factor in determining the reaction specificity of pyridoxal-phosphate-dependent enzymes. We have quantified the contribution of the α-carboxylate to the stereospecificity of α-proton exchange reactions catalysed by tryptophan synthase [2,5,9] and serine hydroxymethyltransferase [2,5], and we are assessing whether the R-group of amino acids also contributes to the stereospecificity of these α-proton-exchange reactions [2,4,5,10].

In studies with serine hydroxymethyltransferase, it was found that as the size of the R-group of the amino acids glycine, aminobutyrate, norvaline and norleucine increased, there was a change in stereospecificity from the preferential exchange of the pro-2S proton of glycine to the preferential exchange of the pro-2R proton of L-amino acids [4]. This change in stereospecificity was shown to result from the larger amino acids being unable to bind at the normal catalytic site, which bound smaller amino acids such as glycine and serine. Instead, these larger amino acids are bound at a second catalytic site, well separated from the normal catalytic site, which binds glycine and serine. It is clear therefore that for serine hydroxymethyltransferase, the type of amino acid R-group plays a major role in determining the stereospecificity of the α-proton-exchange reactions it catalyses. The stereospecificity of > 200 000 for the first-order exchange of the α-proton of L-norleucine at the second site is significantly more than the stereospecificity of 6900 observed for the first-order exchange rate of the pro-2S proton of glycine [2,4]. This contradicts the assumption of Dunathan [12] that binding of the α-carboxylate anion will be the most important factor in determining the reaction specificity of pyridoxal-phosphate-dependent enzymes. However, the first-order exchange rates at the second site were at least 100 times lower than those observed at the normal catalytic site [2,4], showing that this catalytic pathway is not very efficient.

Tryptophan synthase is an αβ2 complex which catalyses the synthesis of L-tryptophan. The α-subunits catalyse the cleavage of indol-3-ylglycerol phosphate to give indole and D-glyceraldehyde 3-phosphate. The β-subunits catalyse the formation of L-tryptophan from L-serine and indole. DL-α-glyceraldehyde 3-phosphate can be considered as an analogue of D-glyceraldehyde 3-phosphate or of indol-3-ylglyceraldehyde. It acts as an allosteric effector by binding to the active site of the α-subunit and inducing a conformational change at the active site of the β-subunit, which increases its affinity for amino acids and promotes loss of the α-proton of the bound amino acid to form a quinonoid intermediate [13]. Binding of DL-α-glyceraldehyde 3-phosphate changes the α-subunit from an open to a closed form where the entrance to the tunnel connecting the α- and β-active sites is blocked, preventing access to the tunnel via the α-active site [14–16]. Other molecules such as serine and tryptophan can access the β-site directly from solution [15,16].

We have shown that the αβ2 tetramer of tryptophan synthase preferentially catalyses the exchange of the pro-2R proton of glycine [5,9] and that in the presence of 50 mM DL-α-glyceraldehyde 3-phosphate, the stereospecificity of the first-order α-proton-exchange reactions increased in the order glycine, alanine and tryptophan [10]. In the present study, we determined whether this effect occurs when tryptophan synthase catalyses exchange in the absence of the allosteric effector, DL-α-glyceraldehyde 3-phosphate, and whether similar effects are observed as the size of the R-group of the amino acids glycine, aminobutyrate, norvaline and

1 To whom correspondence should be addressed (e-mail J.Paul.G.Malthouse@ucd.ie).
noleucine increases. We also estimated the α-proton pKₐ values of these amino acids and determined whether the observed α-proton exchange rates are related to their pKₐ values.

**MATERIALS AND METHODS**

[2-¹³C]Glycine (99 atom %) was obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). ²H₂O (99.9 atom %) was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. All other chemicals used were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

**Isolation and assay of tryptophan synthase and its α and β₂ subunits**

The tryptophan synthase α₂β₂ complex from *Salmonella typhi-
murium* was purified from the *Escherichia coli* strain CB149 that lacks the trp operon and harbours the high-copy plasmid pEBA10 containing the wild-type *trp* A and *trp* B genes from *S. typhi-
murium* (kindly provided by Dr Edith Wilson Miles, National Institutes of Health, Bethesda, MD, U.S.A.) as described previously [17,18]. The α-subunit was isolated from the α₂β₂ enzyme complex by acid precipitation of the β₂-subunit at pH 4.5 [19,20]. The β₂ subunit was isolated from the α₂β₂ enzyme complex by heat denaturation at 63 °C of the α-subunit at pH 7.8 [20,21].

The concentrations of the functional active sites of the isolated β₂-subunits and the β₂-subunits within the α₂β₂ enzyme complex were determined as described by Milne and Malthouse [9]. When the specific activity was less than 6.5 μmol/ml per min, additional α-subunits were added to ensure maximal activity and that no free β₂-subunits were present in the α₂β₂ enzyme samples used.

**NMR samples**

All solutions were made up in 99.9 atom % ²H₂O. Freeze-dried samples of the α₂β₂ enzyme complex and samples of the α- and β₂-subunits were made up in solutions containing 99.9 atom % ²H₂O. For ¹H-NMR studies using L- and D-alanine, serine, aminobutyrate, norvaline, norleucine and tryptophan, the enzyme-cata-
lysed-exchange reactions were initiated by adding 0.01–0.10 ml of enzyme to give a final volume of 0.5 ml. The accumulation of spectra occurred 5–7 min after addition of the enzyme. When the NMR observations were completed, the temperature, pH and catalytic activity of the sample were determined. Except when stated otherwise, all samples were in 0.05 M potassium phosphate buffer at pH 7.8. Sample temperatures were 25 ± 1 °C, and no loss of enzyme activity was detected during the experiments. pH values were determined as described by Malthouse et al. [5].

**NMR spectra**

¹³C-NMR was used to monitor the exchange of the α-protons of [2-¹³C]glycine and ¹H-NMR was used to monitor the exchange of the α-protons of all the other amino acids studied. Spectra were recorded by using a Bruker Avance DRX 500 standard-
bore spectrometer operating at 25.7716 MHz for ¹³C-nuclei and at 500 MHz for ¹H-nuclei. Spin lattice relaxation times (T₁) were determined using the inversion recovery pulse sequence [22]. The T₁s of the α-protons of L-aminobutyrate, L-norvaline, L-norleucine, L-tryptophan and L-serine were 3.9 ± 0.4, 3.5 ± 0.2, 3.0 ± 0.1, 0.69 ± 0.01 and 4.3 ± 0.3 s respectively. The loss of the doublet due to the β₂-protons of alanine was used to monitor the exchange of the α-proton of alanine and its T₁ was 1.2 ± 0.1 s. Samples were in 5-mm-diameter sample tubes. The experimental conditions used were: 32768 time-domain data points; 2.56 s acquisition time; a relaxation delay was used such that the delay between 90° pulses was 5 × T₁; spectral width 12.8 p.p.m.; 1.0 Hz exponential weighting factor and a 90° pulse was used.

**Observation of the hydrogen–deuterium exchange rates of the α-protons of alanine, serine, aminobutyrate, norvaline, norleucine and tryptophan**

1H-NMR was used to determine the extent of hydrogen–deuterium exchange at the α-carbons of L-alanine, L-serine, L-aminobutyrate, L-norvaline, L-norleucine and L-tryptophan. The exchange of the α-proton of L-serine is shown in Figure 1. The exchange of the α-protons of L- and D-serine, aminobutyrate, norvaline, norleucine and tryptophan was monitored by the decrease in the intensity of the doublet of doublets or triplet (centred at 3.6–3.9 p.p.m.) due to the α-proton. For L- and D-alanine, the exchange of the α-proton was monitored by the rate of conversion of the doublet centred at 1.48 p.p.m. into a singlet.

**RESULTS AND DISCUSSION**

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

Scheme 1 represents the minimal kinetic scheme for the trypto-
phan-synthase-catalysed exchange of the α-proton of glycine, alanine, serine, aminobutyrate, norvaline, norleucine and trypto-
phan, and eqn 1 is the differential rate equation for hydrogen–
deuterium exchange according to Scheme 1 [5].

\[
\begin{align*}
\frac{d[SD]}{dt} &= k_s[E][SD][SD]_\infty - [SD] \\
&= \frac{k_s[E][SD][SD]_\infty - [SD]}{[S]_0 + K + C[SD]} \\
k_s &= \frac{k_2(k_4[D] + k_5[H])}{k_2k_3 + k_4k_5 + k_5[H] + k_6[D] + k_3k_5[H] + k_5k_6[D] + k_5k_6[D]}
\end{align*}
\]
Scheme 1 Minimal kinetic scheme for the tryptophan-synthese-catalysed exchange of the α-protons 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 quinonimino intermediate resulting from the loss of either a proton from ESH or a deuteron from ESD.

$C = \frac{k_3(k_3 - k_1)}{k_2k_3 + k_3k_4 + k_5k_6[H] + k_6k_7[H] + k_8k_9[D] + k_9k_1[D]}$

(3)

$K = \frac{k_6(k_6k_7[H] + k_7k_8[H] + k_9k_1[D] + k_9k_1[D])}{k_1(k_2k_3 + k_5k_6[H] + k_6k_7[H] + k_8k_9[D] + k_9k_1[D])}$

(4)

$k_o = \frac{k_6(k_6k_7[D] + k_9k_1[H])}{k_6k_1[H] + k_6k_7[H] + k_9k_3[D] + k_9k_1[D]}$

(5)

The terms $k_1$, $K$ and C are complex assemblies of rate constants (eqns 2–5) [5]. $k_1$ is the first-order rate constant for exchange when the amino acid is bound to the enzyme. $K$ is the apparent dissociation constant for the enzyme–amino acid complex, $k_o/K$ is the second-order rate constant for exchange with the free enzyme and amino acid, and C is a constant for exchange with a specific amino acid and enzyme. For initial rate measurements the term C[SD] is negligible and $k_{obs}$ values were determined using eqn 6 [5].

$$\frac{d[SD]}{dt} = \frac{k_6[E]_0[SD]_0}{[S]_0 + K}$$

(6)

Complete progress curves were analysed using the integrated form (eqn 7) of eqn 1.

$$\frac{1}{[SD]} \ln \left( \frac{[SD]_\infty - [SD]}{[SD]_\infty} \right) = -k_{obs} \left( \frac{t}{[SD]} \right) - D$$

(7)

$k_{obs} = \frac{k_6[E]_0}{1 + xC}, \quad D = \frac{C}{K} \left( \frac{1}{1 + xC} \right) + [S]_0 \left( \frac{1}{1 + xC} \right), \quad x = \frac{[SD]_\infty}{[S]_0}$

x is the fraction of the amino acid (S) whose α-proton has been exchanged. In 99.9 atom % $^2$H$_2$O, essentially 100% exchange was observed and so $x = 1$. By plotting $k_{obs}/[E]_0$ against $[S]_0$, values of $k_o/(1 + C)$, $K/(1 + C)$ and $k_o/K$ are obtained. Using eqn 7 a value of C/$k_o$ can be calculated at each amino acid concentration from the negative intercept on the abscissa ($D/k_{obs} = C/[k_o[E]_0]$). Values of $k_o$, $K$ and C were determined from these values of C/$k_o$, $k_o/(1 + C)$ and $K/(1 + C)$ [5].

When $C < 1$, and provided less than 75% of exchange is followed then the term C[SD] in eqn (1) can be neglected and the experimental data can be analysed using eqn (6) [23]. Therefore at a given substrate concentration, the observed rate constant, $k_{obs} = k_6[E]_0/[S]_0 + K$. By plotting $k_{obs}$/[S]$_0$/[E]$_0$ against [S]$_0$, a conventional hyperbolic plot is obtained which can be analysed in the same way as Michaelis–Menten data [24].

Re-examination of the tryptophan synthase catalysed first-order exchange of the pro-2R protons of glycine by complete progress curves and initial rate methods gave similar $k_o$ values to those determined in earlier studies [9], although the binding constant was 3–4-fold greater when determined by initial rate methods (Table 1). Likewise, the first-order exchange rate of the slowly exchanged pro-2S proton of glycine determined by initial rate measurements was essentially the same as in earlier studies [10]. However, the binding constant was approx. 2 times smaller that determined in earlier studies (Table 1).

The $k_o$ value obtained for the first-order exchange of the α-protons of L-alanine was also essentially the same as that obtained in our earlier studies [9], but there was an approx. 2-fold difference in the binding constant (Table 1). In an earlier limited study [9] of the exchange of the α-proton of D-alanine, we were unable to detect the binding of D-alanine due to the fact that we

Table 1 Kinetic parameters for the exchange of the α-protons of amino acids at pH 7.8 catalysed by the α2β2 enzyme complex of tryptophan synthase

<table>
<thead>
<tr>
<th>R group</th>
<th>2R proton</th>
<th>2S proton</th>
<th>Stereospecificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_a$ (s$^{-1}$)</td>
<td>$k_a/K$ (M$^{-1}$ s$^{-1}$)</td>
<td>C</td>
</tr>
<tr>
<td>H$^+$</td>
<td>3.20</td>
<td>0.054</td>
<td>59.3</td>
</tr>
<tr>
<td>H</td>
<td>2.92</td>
<td>0.036</td>
<td>81.1</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>3.16</td>
<td>0.149</td>
<td>21.2</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>8.6</td>
<td>0.273</td>
<td>31.5</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>8.04</td>
<td>0.120</td>
<td>67.0</td>
</tr>
<tr>
<td>CH$_3$, CH$_2$H</td>
<td>0.22</td>
<td>0.165</td>
<td>1.33</td>
</tr>
<tr>
<td>CH$_3$, CH$_2$, CH$_2$H</td>
<td>0.301</td>
<td>0.0354</td>
<td>0.876</td>
</tr>
<tr>
<td>Indole-CH$_2$</td>
<td>1.7</td>
<td>†</td>
<td>0.53</td>
</tr>
<tr>
<td>Indole-CH$_3$</td>
<td>4.53</td>
<td>0.00086</td>
<td>5250</td>
</tr>
</tbody>
</table>

* Data is that for the pro-2R and pro-2S protons of glycine obtained by [9].
† Data obtained by initial rate methods.
‡ Data from [10].
§ Not determined.
Studies [28] on the $pK_a$ values of the $\alpha$-protons of glycine and glycine derivatives have shown that changing the $\alpha$-substituent from $\text{NH}_3^+$ to $\text{NH}_2$ increases the $pK_a$ of the $\alpha$-proton by 5 $pK_a$ units, whereas changing the $\alpha$-substituent from $\text{COO}^-$ to $\text{COOCH}_3$ decreases the $pK_a$ of the $\alpha$-proton by 8 $pK_a$ units. Using these values, and assuming a linear relationship between the $\alpha$-proton $pK_a$ and the appropriate $\text{Taft}$ substituent constant ($\sigma^*$) [29], we estimate that $\Delta pK_a = -\alpha \Delta \sigma^*$, where $\alpha$ is 1.6–2.2. Using this relationship, we have estimated the $\alpha$-proton $pK_a$ of all the amino acids we have studied by calculating how the $pK_a$ of glycine is changed when one of its $\alpha$-protons is replaced by the appropriate side chain (Table 2). In this way, we estimate that the $\alpha$-proton $pK_a$ values of alanine, aminobutyrate, norvaline, norleucine and tryptophan will all be raised by approx. 1 $pK_a$ unit relative to glycine and serine (Table 2).

$$RCH + \text{HOH}^+ \overset{\text{K}_{\text{a1}}}{\underset{\text{K}_{\text{a2}}}} \text{RC}^- + \text{H}_2\text{O}$$

As the $\alpha$-proton $pK_a$ values are all much greater than the $pK_a$ of water ($pK_a = 15.74$), their $k_{a1}$ values would all be expected to occur at the diffusion-controlled limit and so the first-order rate constants ($k_{a1}$) for proton abstraction would be expected to be inversely proportional to the dissociation constant ($K_{\text{a1}} = k_{a1}/k_{a2}$). Therefore as the $\alpha$-proton $pK_a$ values of alanine, aminobutyrate, norvaline, norleucine and tryptophan are approx. 1 $pK_a$ unit higher than those observed for glycine and serine (Table 1), it is predicted that their exchange rates will be approx. 10 times lower than those observed for glycine and serine.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$R$ group</th>
<th>$pK_a$</th>
<th>$k_a$ (s$^{-1}$)</th>
<th>Relative value $k_a$</th>
<th>$k_a/K$ (M$^{-1}$ s$^{-1}$)</th>
<th>Relative value $k_a/K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine*</td>
<td>H</td>
<td>34</td>
<td>3.16</td>
<td>1</td>
<td>21.2</td>
<td>1</td>
</tr>
<tr>
<td>Alanine</td>
<td>CH$_3$</td>
<td>34.8–35.1</td>
<td>8.04</td>
<td>2.54</td>
<td>67</td>
<td>3.16</td>
</tr>
<tr>
<td>Aminobutyrate</td>
<td>CH$_3$,CH$_2$</td>
<td>35.1–35.6</td>
<td>0.22</td>
<td>0.07</td>
<td>1.33</td>
<td>0.06</td>
</tr>
<tr>
<td>Norvaline</td>
<td>CH$_3$,CH$_2$,CH$_3$</td>
<td>35.0–35.3</td>
<td>0.301</td>
<td>0.095</td>
<td>2.39</td>
<td>0.11</td>
</tr>
<tr>
<td>Norleucine</td>
<td>CH$_3$,CH$_2$,CH$_2$</td>
<td>35.2–35.6</td>
<td>0.031</td>
<td>0.01</td>
<td>0.876</td>
<td>0.04</td>
</tr>
<tr>
<td>Serine</td>
<td>HOCH$_2$</td>
<td>33.8–33.7</td>
<td>0.272</td>
<td>0.086</td>
<td>907</td>
<td>42.78</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Indole-CH$_2$</td>
<td>34.8–35.1</td>
<td>4.53</td>
<td>1.43</td>
<td>5250</td>
<td>248</td>
</tr>
</tbody>
</table>
Comparison of the observed and predicted hydrogen–deuterium exchange rates

If the pro-2R and pro-2S protons of glycine exchanged at the same rate, we would have to divide glycine rates by a statistical factor of 2 if we were comparing them with exchange rates obtained with amino acids with only one α-proton. However, as the tryptophan synthase catalysed exchange rates of the pro-2R and pro-2S protons differ approx. 200-fold, this correction is not required.

The first-order exchange rates \(k_1\) of the 2R protons of amino-butyrate and norvaline are approx. 10 times smaller than that of the pro-2R proton of glycine as predicted on the basis of their \(\alpha\)-proton \(pK_a\) values. However, the 1.4-fold increase with L-tryptophan and the 2.5-fold increase with L-alanine shows that the enzyme is catalysing exchange 14–25 times more efficiently than predicted on the basis of their estimated \(pK_a\) values. The lower \(pK_a\) of the \(\alpha\)-proton of L-serine should result in it exchanging 1.6–2 times more rapidly than the pro-2R proton of glycine. However, its first-order exchange rate is approx. 11 times lower than glycine and so approx. 20 times lower than predicted (Table 2). Likewise, the first-order exchange rate with L-norleucine is approx. 10 times lower than expected (Table 2). Therefore we conclude that tryptophan synthase inhibits the first-order rate of exchange of the \(\alpha\)-protons of both L-serine and L-norleucine relative to the pro-2R proton of glycine and the \(\alpha\)-protons of L-aminobutyrate and L-norvaline. The second-order exchange rates \(k_2/k_1\) of L-aminobutyrate, L-norvaline and L-norleucine are close to the values expected on the basis of their \(pK_a\) values, but the value for L-alanine is approx. 30 times larger than predicted and the tight binding of L-serine and L-tryptophan results in their second-order exchange rates being approx. 40 and 250 times greater respectively than glycine and approx. 20 and 2500 times greater than expected on the basis of their \(\alpha\)-proton \(pK_a\) value (Table 2).

For the 2S protons, the changes in the first- and second-order exchange rates are similar to those observed with the 2R protons (Table 1), except that the values are approx. two orders of magnitude smaller. However, D-serine, unlike L-serine, is not tightly bound to tryptophan synthase, and so its second-order exchange rate is not significantly enhanced relative to the pro-2S proton of glycine (Table 1). Although D-tryptophan is bound approx. 1000-fold tighter than the other D-amino acids, its second-order exchange rate is only approx. 10 times greater than that observed for the other D-amino acids due to the small value of its first-order exchange rate constant (Table 1).

Stereospecificity of the exchange reactions

The stereospecificities of the first-order exchange rates of the \(\alpha\)-protons of all the amino acids except tryptophan and norleucine are similar (Table 1). This shows that for all the amino acids except tryptophan and norleucine, the stereospecificities of the first-order exchange reactions are independent of both the size and electronegativity of the R-group. Changes in the electronegativity of an R-group should not affect the stereospecificity of the first-order exchange rates as it should affect \(\alpha\)-proton exchange rates and the \(pK_a\) values of L- and D-amino acids equally. While this also applies to the second-order reactions, there is more variability in the second-order stereospecificities (Table 1) as they also reflect the differences in binding efficiency for the D- and L-amino acids. As both L-serine and L-tryptophan are the natural substrate and product respectively for tryptophan synthase, it is not surprising that they are tightly bound and have the highest second-order exchange rates and stereospecificities observed (Table 1).

Effect of the length of the amino acid R-group on the exchange rates and stereospecificities of the \(\alpha\)-protons of D- and L-amino acids catalysed by tryptophan synthase and serine hydroxymethyltransferase

For serine hydroxymethyltransferase-catalysed reactions [4], the rate of the first-order exchange reaction of the \(\alpha\)-proton of D-amino acids decreases, while that of the \(\alpha\)-proton of L-amino acids increases as the size of the R-group increases (Figure 3A). Therefore there is a change in stereospecificity when the first-order exchange rates cross over for the D- and L-amino acids (Figure 3A). It has been suggested [4] that this occurs because the larger amino acids cannot bind at the normal catalytic site and so if there is excess pyridoxal phosphate present, they form Schiff bases with the excess pyridoxal 5′-phosphate, and this Schiff base is bound at another catalytic site with a different stereospecificity.

In contrast with tryptophan-synthase-catalysed exchange reactions, increasing the length of the R-group causes similar changes in the first-order exchange rates of the \(\alpha\)-protons of L- and D-amino acids (Figure 3B). Therefore the stereospecificity of the first-order exchange reaction is essentially independent of the length of the R-group. This is consistent with both D- and L-amino acids being bound in a similar way at the same catalytic site on tryptophan synthase. The stereospecificities of the first-order exchange rates of norleucine and tryptophan are significantly different from those of the other amino acids studied (Table 1). It is not surprising that the highest specificity is observed with L-tryptophan, the normal product for tryptophan synthase.
In our earlier studies [10], we concluded that, in the presence of 50 mM DL-α-glycerol 3-phosphate, the stereospecificity of the first-order exchange rates of the α-protons of glycine, alanine and tryptophan increased as the size of the R-group increased. However, it was not clear whether the stereospecificity of the first-order exchange rates of the α-proton of alanine was greater or smaller than that observed for glycine in the absence of 50 mM DL-α-glycerol 3-phosphate [10]. In the present study, we confirm that, in the absence of 50 mM DL-α-glycerol 3-phosphate, the stereospecificity of the first-order exchange rate of the α-proton of alanine is slightly larger than that observed with glycine. Therefore we conclude that, in the absence of 50 mM DL-α-glycerol 3-phosphate, the stereospecificity of the first-order exchange rates of the α-protons of glycine, alanine and tryptophan increased as the size of the R-group increases. However, for all the amino acids except tryptophan and norleucine, the stereospecificities of the first-order exchange reactions are similar (Table 1), showing that for all the amino acids studied, except tryptophan, the stereospecificity of exchange does not increase significantly with the size of the R-group. Indeed with norleucine, the stereospecificity of the first-order exchange rate decreases. Therefore only with tryptophan does the R-group make a significant positive contribution to the stereospecificity of the first-order exchange rates catalysed by tryptophan synthase. Therefore the assumption of Dunathan [12] that the binding of the carboxylate ion would be the most important factor in controlling the reaction specificity of pyridoxal-phosphate-dependent enzymes is correct for the tryptophan-synthase-catalysed first-order exchange of the α-protons of glycine, alanine, aminobutyrate, serine, norvaline and norleucine.

The fact that binding did not improve as the size of the R-groups of glycine, alanine, aminobutyrate, norvaline and norleucine increased (Table 1) most probably reflects the fact that better binding by the larger aliphatic side chains will be offset by the increased (Table 1) most probably reflects the fact that better binding by the larger aliphatic side chains was offset by the increased entropic energy required to freeze out their motions on binding. Such effects are expected to be minimal with the rigid aromatic side chain of tryptophan explaining why it is bound more tightly.

The stereospecificities of the second-order exchange rates were similar (Table 1), except that the natural substrates serine and tryptophan had significantly larger second-order stereospecificities (Table 1). For serine, this was largely due to extremely tight binding of L-serine, whereas for tryptophan, its higher second-order stereospecificity was due to tight binding of L-tryptophan and a greatly reduced first-order exchange rate for the α-proton of D-tryptophan.

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