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

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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 pK_a 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 pK_a values. With the exception of tryptophan and norleucine, the stereo-

specificities of the first-order α -proton exchange rates are independent of the size and electronegativity 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 $\alpha_2\beta_2$ 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 -glycerol 3-phosphate can be considered as an analogue of D-glyceraldehyde 3-phosphate or of indol-3-ylglycerol phosphate. 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 -glycerol 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 $\alpha_2\beta_2$ 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 -glycerol 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 -glycerol 3-phosphate, and whether similar effects are observed as the size of the R-group of the amino acids glycine, aminobutyrate, norvaline and

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norleucine increases. We also estimated the α -proton pK_a values of these amino acids and determined whether the observed α -proton exchange rates are related to their pK_a values.

MATERIALS AND METHODS

[2- 13 C]Glycine (99 atom %) was obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). $^2\text{H}_2\text{O}$ (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 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 pEBA10 containing the wild-type *trpA* and *trpB* genes from *S. typhimurium* (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 $\alpha_2\beta_2$ enzyme complex by acid precipitation of the β -subunit at pH 4.5 [19,20].

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 [20,21].

The concentrations 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]. When the specific activity was less than 6.5 $\mu\text{mol/ml}$ per min, additional α -subunits were added to ensure maximal activity and that no free β -subunits were present in the $\alpha_2\beta_2$ enzyme samples used.

NMR samples

All solutions were made up in 99.9 atom % $^2\text{H}_2\text{O}$. 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 99.9 atom % $^2\text{H}_2\text{O}$. For ^1H -NMR studies using L- and D-alanine, serine, aminobutyrate, norvaline, norleucine and tryptophan, the enzyme-catalysed-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

^{13}C -NMR was used to monitor the exchange of the α -protons of [2- ^{13}C]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 125.7716 MHz for ^{13}C -nuclei and at 500 MHz for ^1H -nuclei. Spin lattice relaxation times (T_1) were determined using the inversion recovery pulse sequence [22]. The T_1 s 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 β -protons of alanine was used to monitor the exchange of the α -proton of alanine and its T_1 was 1.2 ± 0.1 s. Samples were in 5-mm-diameter sample tubes. The experimental conditions used were: 32 768 time-domain data points; 2.56 s

Figure 1 ^1H -NMR spectra of L-serine before and after exchange of the α -proton with deuterium using $\alpha_2\beta_2$ tryptophan synthase

The sample contained 0.05 M potassium phosphate buffer, pH 7.80, and 99.9 atom % $^2\text{H}_2\text{O}$. Spectrum A is of 0.02 M L-serine. Spectrum B is of 0.02 M L-[2- ^2H]serine produced by incubating L-serine with 7.68 μM wild-type $\alpha_2\beta_2$ tryptophan synthase. Data acquisition for spectrum B commenced 7 h after the addition of tryptophan synthase. Both spectra resulted from 65 accumulations using a pulse width of 90°, an acquisition time of 2.56 s and an interpulse delay of 16 s.

acquisition time; a relaxation delay was used such that the delay between 90° pulses was $5 \times T_1$; 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 tryptophan-synthase-catalysed exchange of the α -proton of glycine, alanine, serine, aminobutyrate, norvaline, norleucine and tryptophan, and eqn 1 is the differential rate equation for hydrogen–deuterium exchange according to Scheme 1 [5].

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

$$k_a = \frac{k_2(k_3k_5[\text{D}] + k_4k_6[\text{H}])}{k_2k_3 + k_3k_6 + k_2k_4[\text{H}] + k_4k_6[\text{H}] + k_2k_5[\text{D}] + k_3k_5[\text{D}]} \quad (2)$$

Scheme 1 Minimal kinetic scheme for the tryptophan-synthase-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 quinonoid intermediate resulting from the loss of either a proton from ESH or a deuteron from ESD.

$$C = \frac{k_2(k_6 - k_3)}{k_2k_3 + k_3k_6 + k_2k_4[H] + k_4k_6[H] + k_2k_5[D] + k_3k_5[D]} \quad (3)$$

$$K = \frac{k_2(k_4k_6[H] + k_2k_4[H] + k_2k_5[D] + k_3k_5[D])}{k_1(k_2k_3 + k_3k_6 + k_2k_4[H] + k_4k_6[H] + k_2k_5[D] + k_3k_5[D])} \quad (4)$$

$$\frac{k_a}{K} = \frac{k_1(k_3k_5[D] + k_4k_6[H])}{k_4k_6[H] + k_2k_4[H] + k_2k_5[D] + k_3k_5[D]} \quad (5)$$

The terms k_a , K and C are complex assemblies of rate constants (eqns 2–5) [5]. k_a 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_a/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].

$$+ \frac{d[SD]}{dt} = \frac{k_a[E]_0[SD]_\infty}{[S]_t + K} \quad (6)$$

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

$$\frac{1}{[SD]} \left[\ln \left(\frac{[SD]_\infty - [SD]}{[SD]_\infty} \right) \right] = -k_{\text{obs}} \left(\frac{t}{[SD]} \right) - D \quad (7)$$

$$k_{\text{obs}} = \frac{\frac{k_a[E]_0}{1 + xC}}{\frac{K}{1 + xC} + [S]_t}, \quad D = \frac{\frac{C}{1 + xC}}{\frac{K}{1 + xC} + [S]_t}, \quad x = \frac{[SD]_\infty}{[S]_t}$$

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

The k_a 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 $\alpha_2\beta_2$ enzyme complex of tryptophan synthase

k_{ar} and k_{as} are the first-order rate constants for the exchange of the 2R and 2S protons respectively of the amino acids studied.

R group	2R proton				2S proton			Stereospecificity	
	k_{ar} (s^{-1})	K (M)	k_{ar}/K ($\text{M}^{-1} \cdot \text{s}^{-1}$)	C	k_{as} (s^{-1})	K (M)	k_{as}/K ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$k_{\text{ar}}/k_{\text{as}}$	$(k_{\text{ar}}/K)/(k_{\text{as}}/K)$
H*	3.20	0.054	59.3	< 0.10	0.011	0.041	0.268	291	221
H	2.92	0.036	81.1	0.08	0.0133	0.0175	0.76	220	107
H	3.16	0.149	21.2	†	0.0133	0.0175	0.76	238	27.9
CH ₃ ‡	8.6	0.273	31.5	0.31	§	§	0.0165	§	1910
CH ₃	8.04	0.120	67.0	0.23	0.0211	0.0709	0.297	381	225
CH ₃ CH ₂	0.22	0.165	1.33	0.18	0.00162	0.0618	0.0262	136	50.8
HOCH ₂	0.272	0.0003	907	0.12	0.00148	0.0148	0.10	184	9070
CH ₃ CH ₂ CH ₂	0.301	0.126	2.39	†	0.00237	0.0644	0.0368	127	65.0
CH ₃ CH ₂ CH ₂ CH ₂	0.031	0.0354	0.876	†	0.00169	0.0805	0.021	18.3	41.7
Indole-CH ₂ ‡	1.7	§	§	0.53	0.000125	§	§	13 600	§
Indole-CH ₂	4.53	0.00086	5250	†	0.000008	0.000039	0.208	565 000	25 200

* 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.

Figure 2 The effects of amino acid concentration on the tryptophan-synthase-catalysed hydrogen-deuterium exchange rates of the α -protons of D-alanine and L-tryptophan at pH 7.80

(A) Concentrations of D-alanine of 0.01–0.4 M and tryptophan synthase active-site concentrations of 25.6–76.8 μ M were used. (B) Concentrations of L-tryptophan of 0.0005–0.035 M and tryptophan synthase active-site concentrations of 0.08–2.4 μ M were used. All samples contained 0.05 M potassium phosphate buffer, pH 7.80, and 99.9 atom % $^2\text{H}_2\text{O}$. The continuous lines in (A) and (B) were calculated using $k_{\text{obs}} \cdot [\text{S}]_0/[\text{E}]_0 = k_a \cdot [\text{S}]/([\text{S}]_0 + K)$ and the fitted parameters: $k_a = 0.0211 \pm 0.0012 \text{ s}^{-1}$, $K = 0.0709 \pm 0.0117 \text{ M}$ and $k_a/K = 0.297 \pm 0.052 \text{ M}^{-1} \cdot \text{s}^{-1}$ (A); $k_a = 4.53 \pm 0.06 \text{ s}^{-1}$, $K = 0.000863 \pm 0.000068 \text{ M}$ and $k_a/K = 5245 \pm 417 \text{ M}^{-1} \cdot \text{s}^{-1}$ (B).

only obtained exchange rates at three substrate concentrations. However, in the present study, we determined exchange rates at twelve substrate concentrations. This has enabled us to observe binding (Figure 2A) and so both the first- and second-order rate constants for exchange have been determined (Table 1). As we had better sensitivity at 500 MHz compared with our earlier studies

at 80 MHz [9], we could study exchange at lower tryptophan concentrations, and so we were able to demonstrate saturation kinetics when we observed the exchange of the α -proton of L-tryptophan (Figure 2B) and D-tryptophan (results not shown). Therefore both the first- and second order exchange rates were determined for L- and D-tryptophan (Table 1). The first-order exchange rate of the 2R proton (Table 1) was 2–3-fold larger than estimated in our earlier studies at 80 MHz [9]. However, the tight binding of tryptophan (Table 1) observed in the present study is consistent with earlier studies that employed other techniques [20,25,26].

The first-order exchange rate of the α -proton of L-serine was similar to the value of 0.37 s^{-1} we reported earlier [8]. However, the second-order exchange rate (Table 1) is much smaller than the value we estimated earlier [8] using a much smaller binding constant estimated from spectrophotometric studies [27]. This is consistent with our earlier prediction that binding constants determined by kinetic and spectrophotometric techniques are not equivalent [9].

Estimation of the pK_a values and exchange rates of the α -protons of alanine, serine, aminobutyrate, norvaline, norleucine and tryptophan

Studies [28] on the pK_a values of the α -protons of glycine and glycine derivatives have shown that changing the α -substituent from NH_3^+ to NH_2 increases the pK_a of the α -proton by 5 pK_a units, whereas changing the α -substituent from COO^- to COOCH_3 decreases the pK_a of the α -proton by 8 pK_a units. Using these values, and assuming a linear relationship between the α -proton pK_a and the appropriate Taft substituent constant (σ^*) [29], we estimate that $\Delta\text{pK}_a = -\alpha\Delta\sigma^*$, where α is 1.6–2.2. Using this relationship, we have estimated the α -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 α -protons is replaced by the appropriate side chain (Table 2). In this way, we estimate that the α -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).

As the α -proton pK_a values are all much greater than the pK_a of water ($\text{pK}_a = 15.74$), their k_{-1} values would all be expected to occur at the diffusion-controlled limit and so the first-order rate constants (k_{+1}) for proton abstraction would be expected to be inversely proportional to the dissociation constant ($K_a = k_{-1}/k_{+1}$). Therefore as the α -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 2 Comparison of the first- and second-order α -proton exchange rates of L-amino acids with the calculated pK_a values of their α -protons

Amino acid	R group	pK_a	$k_a (\text{s}^{-1})$	Relative value k_a	$k_a/K (\text{M}^{-1} \cdot \text{s}^{-1})$	Relative value k_a/K
Glycine*	H†	34	3.16	1	21.2	1
Alanine	CH_3	34.8–35.1	8.04	2.54	67	3.16
Aminobutyrate	CH_2CH_2	35.1–35.6	0.22	0.07	1.33	0.06
Norvaline	$\text{CH}_2\text{CH}_2\text{CH}_2$	35.0–35.3	0.301	0.095	2.39	0.11
Norleucine	$\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$	35.2–35.6	0.031	0.01	0.876	0.04
Serine	HOCH_2	33.8–33.7	0.272	0.086	907	42.78
Tryptophan	Indole- CH_2	34.8–35.1	4.53	1.43	5250	248

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_a) of the 2R protons of aminobutyrate and norvaline are approx. 10 times smaller than that of the pro-2R proton of glycine as predicted on the basis of their α -proton pK_a values (Table 2). 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 (Table 2). The lower pK_a of the α -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 α -protons of both L-serine and L-norleucine relative to the pro-2R proton of glycine and the α -protons of L-aminobutyrate and L-norvaline. The second-order exchange rates (k_a/K) 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 α -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 α -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 α -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).

Figure 3 The effects of the length of the R-group on the first-order rate constant for (A) the serine hydroxymethyltransferase and (B) the tryptophan synthase catalysed exchange of the α -protons of L- and D-amino acids

Amino acids (number of carbons): glycine (1), alanine (2), aminobutyrate (3), norvaline (4), norleucine (5). (A) Serine hydroxymethyltransferase: data from Fitzpatrick and Malthouse [4]. (B) Tryptophan synthase: data from Table 1.

Effect of the length of the amino acid R-group on the exchange rates and stereospecificities of the α -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 α -proton of D-amino acids decreases, while that of the α -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 α -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 increase 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 anion 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 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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