A comparative study of the kinetics and stereochemistry of the serine hydroxymethyltransferase- and tryptophan synthase-catalysed exchange of the pro-2*R* and pro-2*S* protons of glycine

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The stereospecificity of the serine hydroxymethyltransferase (EC 2.1.2.1)- and tryptophan synthase (EC 4.2.1.20)-catalysed exchange of the pro-2*R* and pro-2*S* α -protons of glycine was investigated by using ^{13}C n.m.r. The exchange process is described in terms of a minimal four-step mechanism, and a method for analysing the exchange process by complete progress curves is presented. It is shown that serine hydroxymethyltransferase does not have absolute stereospecificity for the pro-2*S*-proton of glycine, but it catalyses the exchange of this proton 7400 times faster than the pro-2*R* proton of glycine. Tryptophan synthase is shown preferentially to catalyse the exchange of the pro-2*R* proton of glycine at a rate 380 times faster than the pro-2*S* proton of glycine. The exchange rates for the rapidly exchanged α -protons of glycine are similar for both enzymes. However, the exchange rates of the slowly exchanged α -protons differ by an order of magnitude. The structural features that may be responsible for the differences in the stereospecificity of the two enzymes are discussed.

INTRODUCTION

Serine hydroxymethyltransferase (EC 2.1.2.1) is a pyridoxal 5'-phosphate-requiring enzyme that uses 5,10-methylenetetrahydrofolate as a C_1 donor for the formation of serine from glycine, and tetrahydrofolate as a C_1 acceptor in the reverse reaction (Schirch, 1982). In order to form L-serine from glycine the pro-2*S* proton of glycine must be replaced by a hydroxymethylene group. It is therefore not surprising that serine hydroxymethyltransferase has been shown to catalyse the exchange of the pro-2*S* proton of glycine (Jordan & Akhtar, 1970; Akhtar *et al.*, 1975). These studies and a more recent study by Shostak & Schirch (1988) did not produce any evidence that serine hydroxymethyltransferase could catalyse the exchange of the pro-2*R* proton of glycine. However, as it can form quinonoid intermediates with L-amino acids (Ulevitch & Kallen, 1977; Hansen & Davis, 1979; Shostak & Schirch, 1988), it must catalyse the removal of the α -proton of L-amino acids, which is equivalent to the pro-2*R* proton of glycine (Shostak & Schirch, 1988). Serine hydroxymethyltransferase is therefore expected to catalyse the exchange of the pro-2*R* proton of glycine, albeit at a lower rate than the pro-2*S* proton of glycine. In the present study we have used ^{13}C n.m.r. to demonstrate that serine hydroxymethyltransferase does catalyse the exchange of the pro-2*R* proton of glycine, and we quantify its stereospecificity for the pro-2*R* and pro-2*S* protons of glycine.

Tryptophan synthase (EC 4.2.1.20) is a pyridoxal 5'-phosphate-requiring enzyme that catalyses the formation of L-tryptophan from L-serine and indol-3-ylglycerol phosphate (Yanofsky & Crawford, 1972; Miles, 1979). It catalyses the exchange of the α -proton of L-tryptophan (Tsai *et al.*, 1978; Miles, 1980) and L-serine (Turner *et al.*, 1985; Roy *et al.*, 1988; Bailey & Malthouse, 1991). This is expected, as all the reactions catalysed by this enzyme result from the cleavage of the α -proton of an L-amino acid substrate (Miles, 1979), which leads to the formation of a quinonoid intermediate. Thus tryptophan synthase is expected to catalyse the exchange of the α -proton of L-amino acids and the equivalent pro-2*R* proton of glycine.

Houben *et al.* (1989) have shown that glycine, L-alanine and L-histidine all form quinonoid intermediates with tryptophan synthase. They have also demonstrated that tryptophan synthase catalyses the exchange of the α -proton of L-histidine, but not the α -proton of D-histidine. This suggests that tryptophan synthase only catalyses the exchange of α -protons with the *R*-configuration. However, they also reported that the enzyme catalysed the exchange of both of the α -protons of glycine but that it catalysed the exchange of one of these about 100 times faster than the other. In the present study we have used ^{13}C n.m.r. to demonstrate that tryptophan synthase preferentially catalyses the exchange of the pro-2*R* proton of glycine, and we have quantified its stereospecificity for the pro-2*R* and pro-2*S* protons of glycine.

The mechanistic implications of the differences in the stereospecificity of serine hydroxymethyltransferase and tryptophan synthase for the α -protons of glycine are discussed in terms of the conformation of the glycine-cofactor Schiff base and in relation to the position of the enzyme group or groups catalysing the exchange reactions.

MATERIALS AND METHODS

Materials

[2- ^{13}C]Glycine (99 atom %) and $^2\text{H}_2\text{O}$ (99.8 atom %) were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. All other chemicals used were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Methods

Isolation and assay of tryptophan synthase. Tryptophan synthase from *Salmonella typhimurium* was purified from *Escherichia coli* strain CB149 ($\Delta\text{trp EDCBA2 hsdR514 supE44 supF58 lacY1 lacU169 galK2 galT22}$) that had been transformed with a high-copy plasmid pSTB7 containing the wild-type *trpA* and *trpB* genes from *S. typhimurium* (kindly provided by Dr. Edith Wilson Miles) as described by Miles *et al.* (1989).

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Tryptophan synthase activity was determined by monitoring the rate of disappearance of indole during the reaction of serine and indole to form tryptophan (Tsai & Suskind, 1972; Bailey & Turner, 1983; Miles *et al.*, 1987). The concentration of active enzyme was determined by activity measurements, assuming that the pure enzyme had a maximum specific activity of $6.5 \mu\text{mol}/\text{mg}$ per min (Miles *et al.*, 1987) and M_r 144000 (Hyde *et al.*, 1988).

All enzyme concentrations are expressed as concentrations of enzyme active sites (1 active site/ β -subunit) given that the α - and β -subunits of the holoenzyme ($\alpha_2\beta_2$) have M_r values of 29000 and 43000 respectively (Hyde *et al.*, 1988).

Isolation of serine hydroxymethyltransferase from bovine liver.

The isolation procedure of Jones & Priest (1976) was modified for the isolation of large quantities of serine hydroxymethyltransferase from bovine liver. All the buffers used contained 10 mM-2-mercaptoethanol and 1 mM-EDTA. Bovine liver (500 g) from a freshly slaughtered animal was added to 700 ml of 0.01 M-sodium phosphate buffer, pH 7.4, to give a total volume of 1.1 litres. This solution was then homogenized in a blender, and solid material was removed by centrifuging at 16300 *g* for 1 h at 4 °C. The supernatant was incubated at 55 °C for 5 min. It was then cooled to 5 °C by the addition of ice. Insoluble material was then removed by centrifugation as before. The supernatant (1.15 litres) was added to 1.8 litres of CM-Sephadex C50 previously equilibrated in 0.01 M-sodium phosphate buffer, pH 6.5, containing 0.1 M-NaCl. The gel was then washed with 7 litres of equilibrating buffer, with a sintered-glass filter to retain the gel. The enzyme was eluted from the gel by using 750 ml of the equilibrating buffer containing 0.7 M-NaCl. Solid $(\text{NH}_4)_2\text{SO}_4$ (390 g/litre) was added to the eluate, and, while stirring, the pH was adjusted to 7.8 with aq. 0.1 M- NH_3 . The precipitated enzyme was collected by centrifuging at 16300 *g* for 1 h at 4 °C. The enzyme was then dissolved in 50 ml of 0.02 M-potassium phosphate buffer, pH 7.8, and this solution was dialysed against 3×4 litres of the same buffer for a period of 24 h.

The enzyme isolated in this way was free of contaminating proteins when analysed by SDS/PAGE, and it was estimated that its M_r was 45000 (Weber & Osborn, 1969; Laemmli, 1970). This value is similar to the value of approx. 50000 obtained by Jones & Priest (1976). The enzyme was assayed with L-allothreonine as substrate as described by Malkin & Greenberg (1964), and the pure enzyme when analysed in this way had a specific activity of $2.6 \mu\text{mol}/\text{mg}$ of protein per min. The concentration of enzyme active sites was determined from activity measurements, assuming that the pure enzyme had M_r 45000 and a maximum specific activity of $2.6 \mu\text{mol}/\text{mg}$ per min when L-allothreonine was used as a substrate. Protein concentrations were determined by the modified method of Lowry *et al.* (1951) as described by Hartree (1972), with BSA as standard.

N.m.r. samples. All solutions were made up in 99.8 atom % $^2\text{H}_2\text{O}$. Freeze-dried tryptophan synthase was dissolved in 99.8 atom % $^2\text{H}_2\text{O}$ containing 0.2 mM-pyridoxal 5'-phosphate and its activity was determined. The exchange reaction was initiated by adding 0.5 ml of the enzyme solution to 0.5 ml of 0.2 M-potassium phosphate buffer, pH 6.8, containing $[2\text{-}^{13}\text{C}]$ -glycine. The accumulation of spectra occurred 4–6 min after addition of the enzyme. When the n.m.r. observations were completed, the sample's temperature, pH* and activity were determined. Samples usually contained 0.1 M-potassium phosphate and 0.1 mM-pyridoxal 5'-phosphate (see Fig. 3).

The same procedure was used for the serine hydroxymethyltransferase-catalysed exchange of $[2\text{-}^{13}\text{C}]$ -glycine except that full anaerobic precautions were taken, e.g. reagents were added from syringes and all samples were kept sealed under an

atmosphere of N_2 . The final reaction mixtures contained 5 mM-2-mercaptoethanol, 0.2 mM-tetrahydrofolate, 0.1 mM-pyridoxal 5'-phosphate and 0.1 M-potassium phosphate buffer.

Sample temperatures were 27 ± 1 °C and no loss of enzyme activity was detected during the experiments. pH* values were recorded at the end of the reaction with an electrode standardized in $^1\text{H}_2\text{O}$ buffers before transfer to $^2\text{H}_2\text{O}$. The pH* of all samples was in the range 6.8–7.0.

N.m.r. spectra. Spectra were recorded by using a Bruker WP80 wide-bore spectrometer at 20.115 MHz for ^{13}C nuclei. Sample size was 1 ml with 10 mm-diameter sample tubes. Typical spectral conditions were: 4000 time-domain data points; 1.27 s acquisition time; spectral width 80 p.p.m.; 2 Hz exponential weighting factor. Under our experimental conditions, $[2\text{-}^{13}\text{C}, ^2\text{H}_2]$ -glycine had a spin-lattice relaxation time, $T_1 = 42$ s. Therefore a pulse

Fig. 1. ^{13}C -n.m.r. time course of the tryptophan synthase-catalysed hydrogen-deuterium exchange of an α -proton of $[2\text{-}^{13}\text{C}]$ -glycine

Experimental conditions were as follows: $[2\text{-}^{13}\text{C}]$ -glycine, 0.43 M; tryptophan synthase active sites, $31.3 \mu\text{M}$; potassium phosphate buffer, pH* 6.95, 0.17 M; pyridoxal 5'-phosphate, 0.1 mM; sample volume, 1 ml. Data acquisition commenced 5.5 min after the reaction was initiated. The spectra (a)–(i) were recorded sequentially; each spectrum resulted from 4096 accumulations (total accumulation time/spectrum = 87 min).

Fig. 2. ^{13}C -n.m.r. time course of the serine hydroxymethyltransferase-catalysed hydrogen-deuterium exchange of the α -proton of a sample of $[2-^{13}\text{C},^2\text{H}_1]\text{glycine}$ prepared by incubation with tryptophan synthase

Experimental conditions were as follows: $[2-^{13}\text{C}]\text{glycine}$, 0.37 M; serine hydroxymethyltransferase active sites, $34.0\ \mu\text{M}$; potassium phosphate buffer, pH* 7.0, 0.37 M; pyridoxal 5'-phosphate, 0.2 mM; 2-mercaptoethanol, 5.0 mM; tetrahydrofolate, 0.2 mM; sample volume, 0.88 ml. Data acquisition commenced 6 min after the reaction was initiated. The spectra (a)–(i) were recorded sequentially; each spectrum resulted from 1024 accumulations (total accumulation time/spectrum = 17.4 min).

width of 1 or 2 μs was used (12 μs = 90° pulse) in all experiments to avoid saturation of signals. The spectra presented (Figs. 1 and 2) were obtained without broadband proton decoupling. However, 1 W broadband decoupling was used to improve the signal-to-noise ratio when the exchange reaction was monitored with low concentrations (0.011–0.25 M) of $[2-^{13}\text{C}]\text{glycine}$ (Fig. 3).

RESULTS AND DISCUSSION

Stereospecificity of the tryptophan synthase-catalysed exchange of the α -proton of glycine

In the absence of proton decoupling, the ^{13}C -enriched carbon atom of $[2-^{13}\text{C}]\text{glycine}$ gives a characteristic triplet ($J_{\text{CH}} = 143.4\ \text{Hz}$). When one of the directly bonded α -protons is replaced by a deuterium atom a doublet of triplets is produced that is due to the coupling of the ^{13}C -enriched carbon atom to

Scheme 1. Minimal kinetic scheme for the serine hydroxymethyltransferase- and 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.

the directly bonded α -deuteron ($J_{\text{CD}} = 22.0\ \text{Hz}$) and α -proton ($J_{\text{CH}} = 143.4\ \text{Hz}$). If both protons are replaced by deuterium, a characteristic quintet ($J_{\text{CD}} = 22.0\ \text{Hz}$) is observed for the ^{13}C -enriched atom of $[2-^{13}\text{C},^2\text{H}_2]\text{glycine}$. Therefore it can be seen (Fig. 1) that tryptophan synthase preferentially catalyses the exchange of one of the protons of $[2-^{13}\text{C}]\text{glycine}$. The same result was obtained when serine hydroxymethyltransferase was incubated with $[2-^{13}\text{C}]\text{glycine}$. However, when serine hydroxymethyltransferase was added to $[2-^{13}\text{C},^2\text{H}_1]\text{glycine}$ prepared by using tryptophan synthase, the second proton was exchanged (Fig. 2) at the same rate as that observed when non-exchanged $[2-^{13}\text{C}]\text{glycine}$ was used. Since it has been shown (Jordan & Akhtar, 1970; Akhtar *et al.*, 1975) that serine hydroxymethyltransferase specifically catalyses the exchange of the pro-2S proton of glycine, then tryptophan synthase must preferentially catalyse the exchange of the pro-2R proton of glycine. Replacement of a proton by deuterium resulted in a deuterium isotope shift of 0.24 p.p.m. at the α -carbon of glycine.

Kinetic analysis of the hydrogen-deuterium exchange reactions of tryptophan synthase and serine hydroxymethyltransferase

Scheme 1 represents a minimal kinetic scheme for enzyme-catalysed exchange of the α -proton of amino acids by tryptophan synthase or serine hydroxymethyltransferase.

The formation of the Schiff bases (ESH and ESD) and their breakdown to form free enzyme and an amino acid does not involve cleavage of the α -proton or -deuteron of the amino acid. Therefore we have assumed that $k_1 = k_8$ and $k_2 = k_7$. Making this assumption, the differential rate equation (eqn. 1) for hydrogen-deuterium exchange was derived by the method of King & Altman (1956):

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

The terms k_a , C and K are complex assemblies of rate constants (see the Appendix). If initial rates are being determined then $[\text{SD}] \sim 0$ and the term $C[\text{SD}]$ is negligible. Therefore eqn. (2) can be used to determine $k_{\text{obs.}}$ ($k_{\text{obs.}} = k_a[\text{E}]_0/([\text{S}]_t + K)$).

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

If there is no deuterium isotope effect on the rate of formation of the quinonoid intermediate ($k_3 = k_6$, Scheme 1) then $C = 0$ (see the Appendix). Likewise, if C is negligible then the integrated form of eqn. (2) can be used to determine $k_{\text{obs.}}$ by a conventional

logarithmic plot. In the present study, complete progress curves have been used to monitor exchange. Since a deuterium isotope effect is expected on the steps (k_3 and k_6) leading to formation of the quinonoid intermediate, we cannot assume that $C = 0$. Therefore the integrated form (eqn. 3) of eqn. (1) was used to analyse the exchange data:

$$\frac{1}{[\text{SD}]} \left[\ln \left(\frac{[\text{SD}]_\infty - [\text{SD}]}{[\text{SD}]_\infty} \right) \right] = -k_{\text{obs.}} \left(\frac{t}{[\text{SD}]} \right) - D \quad (3)$$

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

When exchange data are analysed using eqn. (3), the negative intercept ($D/k_{\text{obs.}}$) on the abscissa is $C/(k_a[\text{E}]_0)$. By using data from ten individual exchange experiments (glycine concentrations 0.011–0.442 M), a mean value of 0.29 ± 0.19 s was obtained for C/k_a . However, the values of $t/[\text{SD}]$ are large when the exchange of low concentrations of glycine is analysed. Therefore, as the values of C/k_a are obtained by extrapolation to the same point on the abscissa, a more accurate estimate of C/k_a is obtained when the data from exchange reactions with high concentrations of glycine are analysed. From four such experiments (glycine: 0.384–0.442 M) a mean value of $C/k_a = 0.348 \pm 0.102$ s was calculated for the tryptophan synthase-catalysed exchange of the pro-2R proton of glycine.

From the substrate-concentration-dependence of $k_{\text{obs.}}/[\text{E}]$ (eqn. 3) it was estimated (Fig. 3) that the first-order rate constant ($k_{\text{obs.}}/[\text{E}]$) for exchange in the presence of saturating concentrations of glycine was 1.06 s^{-1} . As at least 98% of the

Fig. 3. Effect of the concentration of $[2\text{-}^{13}\text{C}]$ glycine on the tryptophan synthase-catalysed exchange of the α -proton of $[2\text{-}^{13}\text{C}]$ glycine

Experimental details were as follows. (1) For $[2\text{-}^{13}\text{C}]$ glycine concentrations of 0.011–0.385 M: tryptophan synthase active sites, 4.5–49 μM ; potassium phosphate buffer, $\text{pH}^* 6.9 \pm 0.1$, 0.1 M; pyridoxal 5'-phosphate, 0.1 mM. (2) For $[2\text{-}^{13}\text{C}]$ glycine concentrations of 0.433, 0.434 and 0.442 M experimental conditions were identical with those in (1) except that potassium phosphate concentrations were 0.17 M, 0.25 M and 0.027 M respectively. The continuous line was calculated by using the equation:

$$\frac{k_{\text{obs.}}}{[\text{E}]} = \frac{\tilde{k}_{\text{obs.}}}{K_{\text{obs.}} + [\text{S}]_t}$$

and the fitted parameters $\tilde{k}_{\text{obs.}} = 1.056 \pm 0.0003 \text{ s}^{-1}$, $K_{\text{obs.}} = 33.55 \pm 0.01 \text{ mM}$ and $\tilde{k}_{\text{obs.}}/K_{\text{obs.}} = k_a/K = 31.47 \pm 0.08 \text{ M}^{-1} \cdot \text{s}^{-1}$. The data were fitted by using a computerized non-linear least-squares regression program (Cleland, 1979).

exchangeable hydrogen atoms were exchanged at equilibrium, we have assumed that $x = 1$ ($x = [\text{SD}]_\infty/[\text{S}]_t$). Therefore, using the relationship $k_{\text{obs.}}/[\text{E}] = k_a/(1 + xC)$ and a value of 0.348 s for C/k_a , we calculated that $k_a = 1.67 \text{ s}^{-1}$ and $C = 0.58$. In the same way, using the relationship $K_{\text{obs.}} = K/(1 + xC)$ we calculated $K = 53 \text{ mM}$. The second-order rate constant $k_a/K = 31.5 \text{ M}^{-1} \cdot \text{s}^{-1}$ was obtained directly by the analysis of the dependence of $k_{\text{obs.}}/[\text{E}]$ on glycine concentration (Fig. 3). Houben *et al.* (1989) obtained a value of $6.4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for $k_{\text{obs.}}/[\text{E}]$ with tryptophan synthase from *E. coli* when the exchange of 0.1 M-glycine was analysed by a conventional first-order analysis. This value of $k_{\text{obs.}}/[\text{E}]$ ($6.4 \text{ M}^{-1} \cdot \text{s}^{-1}$) is similar to the value of $7.86 \text{ M}^{-1} \cdot \text{s}^{-1}$ we obtained for the *S. typhimurium* tryptophan synthase-catalysed exchange of 0.117 M-glycine, which is consistent with the fact that the catalytic properties of the enzymes from both these sources are very similar (Miles *et al.*, 1987). Varying the phosphate concentration from 0.025 to 0.25 M did not affect the exchange rates when 0.434–0.442 M- $[2\text{-}^{13}\text{C}]$ glycine was used (see Fig. 3).

When the data (e.g. Fig. 2) for the serine hydroxymethyltransferase-catalysed exchange of the pro-2S proton of $[2\text{-}^{13}\text{C}]$ glycine was analysed by eqn. (3), it was found that $k_a/C \geq 20 \text{ s}^{-1}$. Consequently, C was negligible and $k_{\text{obs.}}$ values could be determined by using the integrated form of eqn. (2). As C is a ratio of rate constants (see the Appendix), it cannot be assumed that because C is negligible there is no isotope effect on the steps leading to formation of the quinonoid intermediate (see steps k_3 and k_6 , Scheme 1). Schirch & Ropp (1967) have shown that glycine binds tightly to rabbit cytosolic serine hydroxymethyltransferase in the presence of tetrahydrofolate ($K_d = 1.4 \text{ mM}$). Using the same spectrophotometric technique we have estimated that, for the experimental conditions used in our exchange experiments, the apparent K_d is $\leq 13 \text{ mM}$ for the bovine liver enzyme. Therefore, provided that $[\text{S}] \gg K$, then, as C is negligible, k_a can be determined by multiplying $k_{\text{obs.}}/[\text{E}]$ values by the appropriate glycine concentration. In this way it was calculated that $k_a = 4.0 \pm 0.3 \text{ s}^{-1}$ (three experiments, glycine concentrations 0.11–0.39 M). This result is consistent with the value of $> 0.5 \text{ s}^{-1}$ estimated by Shostak & Schirch (1988) for serine hydroxymethyltransferase from *E. coli*.

Degree of stereospecificity of the tryptophan synthase- and serine hydroxymethyltransferase-catalysed exchange of the α -protons of glycine

In the presence of serine hydroxymethyltransferase or tryptophan synthase the second α -proton of glycine was also exchanged. The exchange rate of the second α -proton was at least 100-fold lower than the exchange rate of the first α -proton. Therefore both processes could be treated as kinetically independent events, and the exchange rate of the slowly exchanged proton was determined from initial-rate measurements by using eqn. (2). There was no evidence for the exchange of either of the α -protons of glycine in control samples that did not contain either enzyme. The first-order rate constant of 0.000817 s^{-1} for the removal of the α -proton of L-phenylalanine by serine hydroxymethyltransferase (Ulevitch & Kallen, 1977) is essentially the same as that for the exchange of the equivalent pro-2R proton of glycine (Table 1). Thus the fact that serine hydroxymethyltransferase can catalyse the removal of the α -proton of L-phenylalanine is not anomalous, as was previously suggested (Ulevitch & Kallen, 1977; Hansen & Davis, 1979; Schirch, 1982). However, the exchange rate of the α -proton of L-alanine (Shostak & Schirch, 1988) is two orders of magnitude greater than that of the equivalent pro-2R proton of glycine (Table 1). This may be due to the L-alanine-cofactor Schiff base being bound differently to the natural glycine-cofactor Schiff base.

Table 1. First order-exchange rates for serine hydroxymethyltransferase- and tryptophan synthase-catalysed exchange of the α -protons of [2- ^{13}C]glycine

k_{af} and k_{as} are the first-order rate constants for rapidly and slowly exchanged protons respectively.

Enzyme	k_{a} (s^{-1})		$k_{\text{af}}/k_{\text{as}}$
	Pro-2S proton	Pro-2R proton	
Tryptophan synthase	0.00440	1.67	380
Serine hydroxymethyltransferase	4.00	0.000538	7400

The ratio ($k_{\text{af}}/k_{\text{as}}$) of the first-order rate constants for the fast exchange process (k_{af}) and the slow exchange process (k_{as}) gives a direct measure of the degree of stereospecificity of both enzymes for the pro-2R and pro-2S protons of glycine (Table 1). Both enzymes catalyse the rapid exchange of one of the α -protons of glycine at similar rates (Table 1). Therefore the differences in the degree of stereospecificity manifested by these enzymes depends primarily on the exchange rate of the slowly exchanged α -proton of glycine (Table 1).

Mechanism of the serine hydroxymethyltransferase- and tryptophan synthase-catalysed exchange of the α -protons of glycine

Dunathan (1966) has argued that for optimal catalysis by pyridoxal 5'-phosphate-dependent enzymes the α -carbon bond that is cleaved must be perpendicular to the plane of the imine-cofactor π -electron system. Consequently, the stereospecificity of bond cleavage depends on the orientation of the amino acid carboxylate and R groups to the plane of the cofactor ring (Dunathan, 1966, 1971). Therefore, the α -protons of glycine that are rapidly exchanged by both enzymes should be orthogonal to the plane of the cofactor ring. Since the proton transfers occur on the same exposed Si face of the cofactor ring (Voet *et al.*, 1973; Dunathan & Voet, 1974; Emery & Akhtar, 1987), the slowly exchanged α -protons must have the opposite geometrical orientation (Fig. 4). With tryptophan synthase a single suprafacial base catalyses the intramolecular transfer of the orthogonal α -proton both to the 4'-carbon atom of pyridoxal 5'-phosphate and to the β -carbon atom of L-serine (Dunathan & Voet, 1974; Tsai *et al.*, 1978). Therefore, as the slowly exchanged pro-2S proton [structure (b), Fig. 4] occupies the same position

as the β -carbon atom of L-serine, we suggest that with tryptophan synthase the base B [structure (b), Fig. 4] should be able to catalyse the exchange of both of the α -protons of glycine. However, if the base B is in an equivalent position in serine hydroxymethyltransferase it will not be able to catalyse the exchange of the pro-2R proton of glycine as efficiently because the pro-2R proton will point away from the base B [structure (a), Fig. 4]. Therefore this model explains why the differences in specificity of the two enzymes are mainly manifested as a difference in the exchange rates of the slowly exchanged α -protons of glycine. The argument for both enzymes having a base B in an equivalent position is supported by the finding that they both bind the same face of the amino acid-cofactor Schiff base and that they catalyse similar suprafacial proton-transfer reactions (Dunathan & Voet, 1974; Emery & Akhtar, 1987). The suggestion that the base B may be the ϵ -amino group of the active-site lysine residue that forms a Schiff base with pyridoxal 5'-phosphate in both tryptophan synthase (Hyde *et al.*, 1988; Miles *et al.*, 1989) and serine hydroxymethyltransferase (Shostak & Schirch, 1988) is also consistent with the base B being in an equivalent position in both enzymes. In the above mechanism we have assumed that for both enzymes the α -carboxylate group of the glycine-cofactor Schiff base can only bind at one site on each enzyme such that the rapidly exchanged α -proton is perpendicular to the plane of the imine-cofactor π -electron system. If, however, the α -carboxylate group can bind at two sites such that either of the α -protons is orthogonal to the cofactor ring, then the specificity of these enzymes for the α -protons of glycine would depend on the populations of these two binding modes. Although this mechanism cannot be dismissed, we favour our earlier mechanism.

Shostak & Schirch (1988) have proposed that in serine hydroxymethyltransferase there are two bases that lie on opposite sides of the pyridoxal 5'-phosphate ring and catalyse the exchange of the α -protons of D- and L-alanine. In view of the high stereospecificity of the enzyme for the pro-2S proton of glycine (Table 1), we see no reason to invoke the presence of a second base to promote the exchange of the slowly exchanged pro-2R proton of glycine.

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Fig. 4. Structures of the glycine-pyridoxal 5'-phosphate Schiff bases formed with (a) serine hydroxymethyltransferase and (b) tryptophan synthase

In these structures it has been assumed that the phenolic hydroxy group of pyridoxal 5'-phosphate is *cis* to the nitrogen atom of the Schiff base as in tryptophan synthase (Hyde *et al.*, 1988).

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APPENDIX

The method of King & Altman (1956) was used to derive eqn. (A1), which is the differential rate equation for hydrogen–

deuterium exchange according to Scheme 1 of the main paper, except that it was assumed that $k_1 = k_8$ and $k_2 = k_7$ (see the Results and discussion section of the main paper):

$$+ \frac{d[\text{SD}]}{dt} = \frac{k_1 k_2 \{k_3 k_5 [\text{D}][\text{S}]_t - [\text{SD}](k_3 k_5 [\text{D}] + k_4 k_6 [\text{H}])\} [\text{E}]_0}{k_2 (k_4 k_6 [\text{H}] + k_2 k_4 [\text{H}] + k_2 k_5 [\text{D}] + k_3 k_5 [\text{D}]) + k_1 (k_2 k_3 + k_2 k_4 [\text{H}] + k_3 k_5 [\text{D}] + k_3 k_6 + k_2 k_5 [\text{D}] + k_4 k_6 [\text{H}]) [\text{S}]_t + k_1 k_2 [\text{SD}](k_6 - k_3)} \quad (\text{A1})$$

At equilibrium there is no net isotope exchange and $+d[\text{SD}]/dt = 0$ and $[\text{SD}] = [\text{SD}]_\infty$. Thus for these conditions we can obtain the following expression (eqn. A2) for $[\text{SD}]_\infty$ from eqn. (A1):

$$[\text{SD}]_\infty = \frac{k_3 k_5 [\text{D}][\text{S}]_t}{k_3 k_5 [\text{D}] + k_4 k_6 [\text{H}]} \quad (\text{A2})$$

Substituting eqn. (A2) in eqn. (A1) and rearranging gives eqn. (A3):

$$\frac{+d[\text{SD}]}{dt} = \frac{k_a [\text{E}]_0 ([\text{SD}]_\infty - [\text{SD}])}{[\text{S}]_t + K + C[\text{SD}]} \quad (\text{A3})$$

where

$$k_a = \frac{k_2 (k_3 k_5 [\text{D}] + k_4 k_6 [\text{H}])}{k_2 k_3 + k_3 k_6 + k_2 k_4 [\text{H}] + k_4 k_6 [\text{H}] + k_2 k_5 [\text{D}] + k_3 k_5 [\text{D}]} \quad (\text{A3a})$$

$$C = \frac{k_2 (k_6 - k_3)}{k_2 k_3 + k_3 k_6 + k_2 k_4 [\text{H}] + k_4 k_6 [\text{H}] + k_2 k_5 [\text{D}] + k_3 k_5 [\text{D}]} \quad (\text{A3b})$$

$$K = \frac{k_2 (k_4 k_6 [\text{H}] + k_2 k_4 [\text{H}] + k_2 k_5 [\text{D}] + k_3 k_5 [\text{D}])}{k_1 (k_2 k_3 + k_3 k_6 + k_2 k_4 [\text{H}] + k_4 k_6 [\text{H}] + k_2 k_5 [\text{D}] + k_3 k_5 [\text{D}])} \quad (\text{A3c})$$

$$\frac{k_a}{K} = \frac{k_1 (k_3 k_5 [\text{D}] + k_4 k_6 [\text{H}])}{k_4 k_6 [\text{H}] + k_2 k_4 [\text{H}] + k_2 k_5 [\text{D}] + k_3 k_5 [\text{D}]} \quad (\text{A3d})$$

If $[\text{SD}] = 0$ at $t = 0$, then integration of eqn. (A3) gives eqn. (A4):

$$\frac{1}{[\text{SD}]} \left[\ln \left(\frac{[\text{SD}]_\infty - [\text{SD}]}{[\text{SD}]_\infty} \right) \right] = - \left(\frac{k_a [\text{E}]_0}{K + [\text{S}]_t + C[\text{SD}]_\infty} \right) \frac{t}{[\text{SD}]} - \frac{C}{K + [\text{S}]_t + C[\text{SD}]_\infty} \quad (\text{A4})$$

If $\frac{1}{[\text{SD}]} \left[\ln \left(\frac{[\text{SD}]_\infty - [\text{SD}]}{[\text{SD}]_\infty} \right) \right]$ is plotted against $\frac{t}{[\text{SD}]}$

$$-\text{Slope} = \frac{k_a [\text{E}]_0}{K + [\text{S}]_t + C[\text{SD}]_\infty} = k_{\text{obs.}} \quad (\text{A5})$$

If x is the fraction of substrate exchanged at equilibrium then $[\text{SD}]_\infty = x[\text{S}]_t$. Substituting for $[\text{SD}]_\infty$ in eqn. (A5) and rearranging gives eqn. (A6):

$$k_{\text{obs.}} = \frac{\frac{k_a}{1 + xC} [\text{E}]_0}{\frac{K}{1 + xC} + [\text{S}]_t} \quad (\text{A6})$$

Values of $k_a/(1 + xC)$, $K/(1 + xC)$ and k_a/K can be obtained from analysis of the substrate dependence of $k_{\text{obs.}}$ (see the Results and discussion section of the main paper).

When $t/[\text{SD}] = 0$ the negative intercept on the abscissa is $C/(k_a [\text{E}]_0)$ and the ratio C/k_a can be calculated. If the values of x and C/k_a are known then k_a , C and K can be calculated provided the values of $k_a/(1 + xC)$ and $K/(1 + xC)$ have been determined (see the Results and discussion section of the main paper).

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