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Kinetic Mechanism of the Interaction of D-Cycloserine with Serine Hydroxymethyltransferase†

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ABSTRACT: The kinetic mechanism for the interaction of D-cycloserine with serine hydroxymethyltransferase (EC 2.1.2.1) from sheep liver was established by measuring changes in the activity, absorbance, and circular dichoism (CD) of the enzyme. The irreversible inhibition of the enzyme was characterized by three detectable steps: an initial rapid step followed by two successive steps with rate constants of 5.4×10^{-3} s^{-1} and 1.4×10^{-4} s^{-1}. The first step was distinguished by a rapid disappearance of the enzyme absorbance peak at 425 nm, a decrease in the enzyme activity to 25% of the uninhibited velocity, and a lowering of the CD intensity at 432 nm to about 65% of the original value. The second step of the interaction was accompanied by a complete loss of enzyme activity and a marginal increase in the CD intensity at 432 nm. The final step resulted in the complete loss of the enzyme absorbance at 425 nm and of the CD band at 432 nm. The products of the reaction were identified as (a) apoenzyme by absorbance measurements, CD spectra, and reconstitution with pyridoxal 5′-phosphate and (b) a pyridoxal 5′-phosphate–D-cycloserine Schiff's base complex identified by its fluorescence and absorbance spectra. The Schiff base complex was expelled from the enzyme active site in the final step of the reaction. The proposed mechanism, which is different from those operative in other pyridoxal phosphate dependent enzymes, probably accounts for the selective inhibition of serine hydroxymethyltransferase by the drug in vivo.

The study of specific irreversible inhibition of pyridoxal phosphate (PLP)[1] dependent enzymes has attracted considerable attention because of the potential therapeutic value of these inhibitors and also in view of their usefulness as probes to study the physiological role of these enzymes (Bey, 1981). The antibiotic D-cycloserine (oxamycin, 4-amino-3-isoxazolidinone) used in the therapy of tuberculosis interferes with cell wall synthesis in the pathogenic bacteria by competitively inhibiting the enzyme D-alanylalanine synthetase (EC 6.3.2.4) (Strominger et al., 1960; Nehaus & Lynch, 1964). D-Cycloserine and its substituents in carbon 5 are rigid cyclic analogues of D-alanine and its higher homologues, respectively (Figure 1). These compounds slowly and irreversibly inhibit several PLP-dependent enzymes following an initial selective and reversible binding as quasi-substrates (Khomutov et al., 1968). Bukin & Sergeev (1968) reported that D-cycloserine selectively inhibited serine hydroxymethyltransferase (EC 2.1.2.1) in mouse liver extracts and in the liver of mice receiving a diet deficient in PLP. The pronounced antineoplastic activity of D-cycloserine and its dimer (Bukin et al., 1970; Sergeev et al., 1971; Draudin-Krylenko, 1976; Bukin & Draudin-Krylenko, 1980), especially in combination with 4-vinylpyridoxal (Bukin et al., 1979), suggested that serine hydroxymethyl transferase might play a critical role in maintaining neoplasia (Bukin & Draudin-Krylenko, 1980).

Although extensive investigations were carried out on the interaction of D-cycloserine with several PLP-dependent enzymes (Braunstein et al., 1961; Dann & Carter, 1964; Karpeiskii et al., 1964; Brown et al., 1969; Roze & Strominger, 1966; Wang & Walsh, 1978), very little information is available on the mechanism of interaction of this drug with serine hydroxymethyltransferase. This enzyme was isolated from several sources and was a homotetramer with a M_r of around 200000 ± 20000 and contained 1 mol of PLP/monomer (Schirch, 1982). We had earlier described the purification of the sheep liver enzyme to homogeneity and described some of its physicochemical, kinetic, and regulatory properties (Manohar et al., 1982). In this paper, we report the kinetic mechanism of the interaction of D-cycloserine with sheep liver serine hydroxymethyltransferase.

Experimental Procedures

Materials

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, MO: 2-mercaptoethanol (2-ME), PLP, ethylenediaminetetraacetic acid (EDTA), D-cycloserine, pyridoxamine 5′-phosphate hydrochloride (PMP), and L-cysteine. L-[3-^{14}C]Serine (58.5 mCi/mmol) was purchased from New England Nuclear, Boston, MA; tetrahydrofolate (H_4-folate) was prepared by the method of Hatefi et al. (1959);

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; EDTA, ethylenediaminetetraacetic acid disodium salt; PMP, pyridoxamine 5′-phosphate hydrochloride; H_4-folate, tetrahydrofolate; CM, carboxymethyl; M_r, molecular weight; 2-ME, 2-mercaptoethanol; UV, ultraviolet; CD, circular dichroism; DTT, dithiothreitol.

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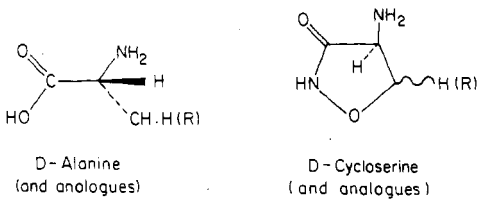

FIGURE 1: Structure of D-cycloserine and D-alanine.

Whatman glass microfiber filter GF/C was purchased from Whatman Ltd., Poole, England.

Methods

Enzyme Purification. Serine hydroxymethyltransferase from sheep liver was purified to homogeneity as described earlier (Manohar et al., 1982) by ammonium sulfate fractionation, carboxymethyl-Sephadex chromatography, gel filtration on Ultrogel AcA34, and Blue Sepharose chromatography. The purified enzyme had a specific activity of 6 ± 0.2 units/mg of protein (1 unit of enzyme activity being defined as the amount of enzyme that catalyzes the formation of 1 μmol of formaldehyde from L-serine and H_4-folate per min at 37 °C and pH 7.4).

Preparation of Holo- and Apoenzymes. The holoenzyme used in all the experiments was obtained by dialyzing the sheep liver serine hydroxymethyltransferase (10 mg/5 mL) against 500 mL of 0.05 M potassium phosphate buffer, pH 7.1, containing 1 mM 2-ME and 1 mM EDTA. The buffer was changed 3 times at 6-h intervals. The apoenzyme was prepared by treating the holoenzyme (2 mg/mL) with L-cysteine (100 mM) and ammonium sulfate (30%) followed by dialysis (Jones & Priest, 1976). This enzyme preparation had no detectable (<0.005) absorbance in the visible region.

Enzyme Assay. The enzyme was assayed by the method of Taylor & Weissbach (1965) with some modifications (Manohar et al., 1982) by using L-[3-^{14}C]serine as substrate. The holoenzyme (0.06 mg/mL) was incubated with D-cycloserine (2.5 mM) in 0.05 M potassium phosphate buffer, pH 7.1, containing 2 mM 2-ME and 1 mM EDTA at 22 °C. At different time intervals in the range 0.5–30 min, aliquots (10 μL) were withdrawn into the reaction mixture containing 0.4 M potassium phosphate buffer, pH 7.1, 1 mM 2 ME, 1 mM EDTA, 3.6 mM L-[3-^{14}C]serine containing 6.6 × 10^5 cpm, 1.8 mM DTT, and 1.8 mM H_4-folate in a final volume of 100 μL. After incubation at 22 °C for 2 min, the reaction was stopped by the addition of 0.1 mL of dimethylcyclohexanedione (0.4 M in 50% ethanol) followed by heating at 90 °C for 5 min. The formaldehyde adduct was extracted into 3 mL of toluene, and the radioactivity was estimated on Beckman LS 100 C liquid scintillation spectrometer.

Absorption Spectroscopy. Absorption spectra were recorded in a Cary 219 double-beam spectrophotometer, at 22 °C in 0.05 M potassium phosphate buffer, pH 7.1, containing 1 mM 2-ME and 1 mM EDTA, against the same buffer. The concentration of the enzyme was 1.5 mg/mL.

Circular Dichroism (CD) Spectroscopy. CD measurements were made in a Jasco J20C automated recording spectropolarimeter, calibrated with *d*-10-camphorsulfonic acid (Cassim & Yang, 1969). The spectropolarimeter was continously purged with pure nitrogen before and during the experiments. Slits were programmed to yield a 10-Å bandwidth at each wavelength.

For the time course of visible CD change, the wavelength was fixed at 432 nm and the chart run at a speed of 0.2 cm/min. For the enzyme, the CD spectra were plotted as molar ellipticity ($[\theta]_{molar}$) by assuming an M_r of 210 000 and by using the formula $[\theta]_{molar} = [\theta]M_r/(10lc)$, where $[\theta]$ is the observed ellipticity in degrees, l is the optical path in centimeters, and c is the concentration of the enzyme in grams per milliliter. For the interactions of free PLP, $[\theta]_{molar}$ was calculated from an M_r of 247.

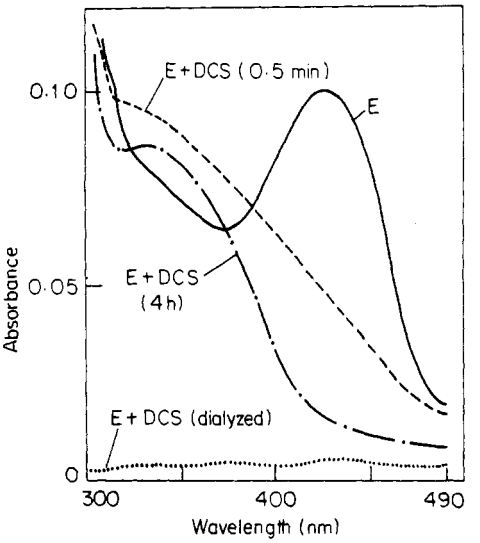

FIGURE 2: Visible absorbance spectra of the holoenzyme (1.5 mg/mL; E), of the holoenzyme at 0.5 min and 4 h after the addition of D-cycloserine (2.5 mM; E + DCS) at 22 °C and pH 7.1, and of the holoenzyme and D-cycloserine after dialysis for 12 h [E + DCS (dialyzed)].

All the CD spectra were recorded at 22 °C in 0.05 M potassium phosphate buffer, pH 7.1, containing 1 mM 2-ME and 1 mM EDTA by using the same buffer as blank. Protein was taken at a concentration of 1.5 mg/mL.

Fluorescence Spectroscopy. The visible fluorescence spectra were obtained in a Hitachi Perkin-Elmer spectrofluorometer. The spectra were taken at 22 °C with an enzyme concentration of 1.5 mg/mL in 0.05 M potassium phosphate buffer, pH 7.1, containing 1 mM 2-ME and 1 mM EDTA. The same buffer was used as a blank.

Results

Absorption Spectra. The holoenzyme had an absorbance maximum at 425 nm (Figure 2) due to bound PLP. Upon the addition of D-cycloserine (2.5 mM) to the holoenzyme, the peak at 425 nm disappeared rapidly (in <0.5 min) with the formation of a shoulder in the region of 330 nm. Following this rapid change was a further slow decrease in the absorbance at 425 nm. This slow decrease was almost complete at the end of 4 h at 22 °C, with the appearance of a new peak of absorbance at 335 nm (Figure 2).

When the absorbance at 425 nm of the holoenzyme 1 min after being mixed with various concentrations of D-cycloserine was plotted as a function of the concentration of D-cycloserine (Figure 3) a hyperbolic pattern was obtained, and maximal change was observed at about 2.5 mM D-cycloserine (Figure 3). Increasing the concentration of D-cycloserine beyond 2.5 mM did not increase the absorbance change. This concentration of D-cycloserine was therefore chosen in all the further experiments. The absorbance change of the holoenzyme, at any of the concentrations of D-cycloserine shown in Figure 3, could not be reversed by decreasing the concentration of the inhibitor by dilution or by the addition of a large excess (25 mM) of L-serine.

When the enzyme (5 mL) incubated with 2.5 mM D-cycloserine for 4 h at 22 °C was dialyzed against 500 ml of 0.05 M potassium phosphate buffer, pH 7.1, containing 1 mM

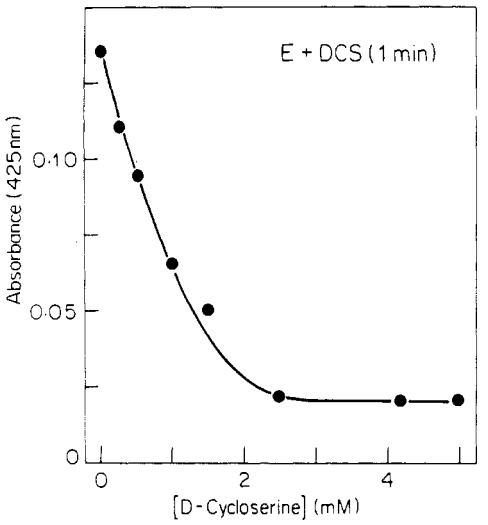

FIGURE 3: Changes in the absorbance of the holoenzyme (1.5 mg/mL) at 425 nm upon incubation with various concentrations of D-cycloserine for 1 min at 22 °C and pH 7.1.

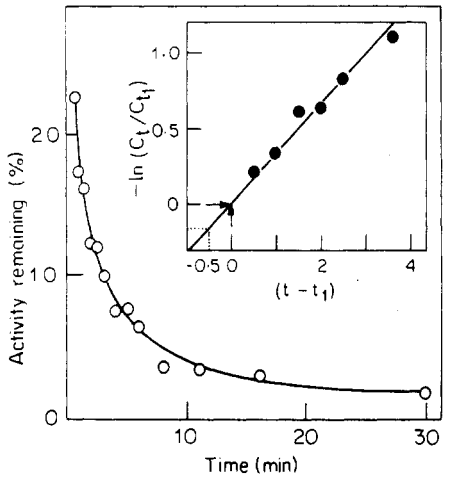

FIGURE 4: Time course of irreversible inhibition of the holoenzyme by D-cycloserine. At time intervals indicated in the figure (0.5–30 min), aliquots were withdrawn from an incubation mixture containing the enzyme (0.06 mg/mL) and D-cycloserine (2.5 mM) and assayed for residual enzyme activity as described under Methods. The velocity of the enzyme in the absence of the inhibitor was normalized to 100, and the residual enzyme activity in the presence of the inhibitor was expressed as percent of this activity. (Inset) First-order replot. C_{t_1} is the activity at time t_1 (0.5 min), and C_t is the activity at time t.

EDTA and 1 mM 2-ME with 3 changes of buffer at 6-h intervals, the absorbance in the visible region reached near zero values (Figure 2). The ultraviolet (UV) absorption spectrum of the D-cycloserine-treated and dialyzed enzyme was very similar to that of the holoenzyme and the apoenzyme prepared by the standard procedure with L-cysteine in Figure 4. All three spectra had a peak at 278 nm. The D-cycloserine- and cysteine-treated enzymes showed a trough at 250 nm, while the holoenzyme had a trough at 254 nm. The ratio of the absorbance at the peak to that at the trough was calculated to be 1.7 for the holoenzyme and 2.3 for both the apoenzyme and the D-cycloserine-treated and dialyzed enzyme. On addition of PLP (0.25 mM) to the D-cycloserine-treated and dialyzed enzyme, it regained the spectral characteristics of the holoenzyme, viz., an absorbance peak at 425 nm, in a manner similar to the apoenzyme prepared by L-cysteine treatment. These results indicate that D-cycloserine interaction might have resulted in the loss of PLP from the enzyme leading to the formation of the apoenzyme.

Free PLP, on the other hand, had an absorbance maximum at 388 nm and a minor peak at 335 nm. When increasing concentrations (0.5–5 mM) of D-cycloserine were added to a solution of PLP (0.25 mM), the peak at 388 nm was diminished, accompanied by an increase in the peak intensity at 335 nm. Thus, the product of the reaction of D-cycloserine with both free and enzyme-bound PLP had absorbance maxima at 335 nm. PMP, a transamination product of the reaction of several PLP enzymes with amino acids, had an absorption maximum at 325 nm.

Enzyme Activity. To measure the irreversible inactivation of the enzyme, the holoenzyme was incubated with 2.5 mM D-cycloserine at 22 °C, and at different intervals of time (0.5–30 min), aliquots were withdrawn and assayed for enzyme activity with L-[3-^{14}C]serine (see Methods). The final concentration of D-cycloserine in the assay was 0.25 mM. This concentration of D-cycloserine did not produce any significant inhibition of the enzyme activity under the assay conditions and in the duration of the assay (2 min). The observed inhibition was therefore regarded as that produced by the inhibitor bound irreversibly to the enzyme in the incubation mixture. Figure 4 represents the time course of the irreversible inhibition of the holoenzyme by 2.5 mM D-cycloserine. It can be seen that even at the earliest time point, viz., 0.5 min, there was a steep fall in the enzyme activity to about 22% of the original activity and, thereafter, it decreased to zero in about 30 min. It was ensured that concentrations of D-cycloserine beyond 2.5 mM did not produce any further inhibition of the enzyme activity. A replot of $-\ln (C_t/C_{t_1})$, vs. $t - t_1$, where C_{t_1} is the enzyme activity at time t_1 (0.5 min) and C_t is the activity at time t, gave a straight line passing through the origin (inset to Figure 4), from the slope of which a first-order rate constant of 5.4×10^{-3} sec^{-1} was calculated. Extrapolation of the first-order plot in Figure 4 to −0.5 min, i.e., the zero time of the reaction, gave an enzyme activity of 27% of the control. The enzyme treated with D-cycloserine for 4 h and dialyzed was inactive. Upon replenishing the enzyme with PLP, activity was restored to that of the native enzyme (sp act. ~6.0).

When the holoenzyme was preincubated with 25 mM L-serine and then reacted with D-cycloserine, a large irreversible inhibition of the enzyme was obtained, but the inhibition was less at each time point than in the absence of serine (70 and 78% as compared with 82 and 88% at 1 and 2 min, respectively). On the other hand, the addition of 25 mM L-serine 0.5 min after the addition of D-cycloserine (2.5 mM) did not alter the inhibition by D-cycloserine.

Circular Dichroic Spectra. The holoenzyme had a prominent positive visible CD band at 432 nm due to the enzyme-bound PLP (Figure 5). The apoenzyme had no CD in this region. It is apparent from Figure 6 that, on adding D-cycloserine (2.5 mM) to the enzyme, an initial sudden decrease in CD intensity at 432 nm to about 65% occurred, followed by a marginal increase up to about 8 min. Although the latter change is experimentally significant, it is too small for kinetic analysis. Following these changes, the CD band at 432 nm decreased slowly to almost the basal level at the end of about 4 h at 22 °C (Figure 6). From the data in Figure 6 the first-order rate constant for the CD change was obtained by plotting $-\ln (D_t/D_{t_1})$ vs. $t - t_1$, where t is an initial time point (48 min), D_{t_1} is the peak intensity at t_1, and D_t is the peak intensity at t (Figure 6, inset). This plot gave a straight line passing through the origin, and from the slope of this line, a first-order rate constant of 1.4×10^{-4} s^{-1} was calculated. By extrapolation of this line to −48 min, i.e., the true zero time, a CD intensity of 76% of the original intensity was calculated.

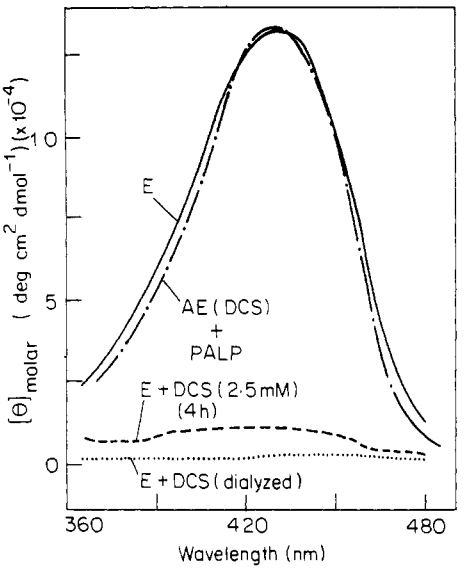

FIGURE 5: Visible CD spectrum of the holoenzyme (1.5 mg/mL; E), of the holoenzyme (1.5 mg/mL) after 4 h of incubation with D-cycloserine (2.5 mM; E + DCS), of the D-cycloserine-treated and dialyzed enzyme [E + DCS (dialyzed)], and of the D-cycloserine-treated and dialyzed enzyme reconstituted with PLP [0.25 mM; AE(DCS) + PLP].

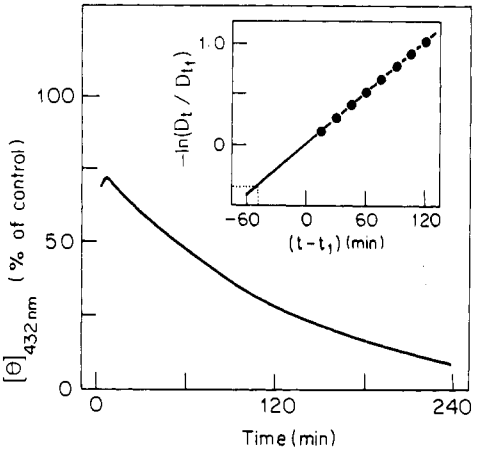

FIGURE 6: Time course of the change in CD intensity (at 432 nm) of the holoenzyme (1.5 mg/mL) upon the addition of D-cycloserine (2.5 mM) at 22 °C and pH 7.1. (Inset) First-order replot. t_1 is an initial point (48 min); D_{t_1} is peak intensity at t_1; D_t is peak intensity at time t.

The near-UV CD spectrum of the holoenzyme also changed slowly after the addition of 2.5 mM D-cycloserine with the negative peaks due to the aromatic residues being generally retracted toward the base line so that at the end of 4 h the spectrum resembled that of the apoenzyme (Figure 7) prepared by the standard procedure from L-cysteine (Jones & Priest, 1976). Upon replenishment with PLP (0.25 mM), both the apoenzyme and the D-cycloserine-treated (4 h) and dialyzed enzyme acquired the visible (Figure 5) and near-UV (Figure 7) CD characterstics of the holoenzyme.

Free PLP had no significant CD band in the visible and near-UV regions. However, the addition of D-cycloserine (0.5 and 2.5 mM) to free PLP (0.25 mM) induced negative CD bands, a weak one at 415 nm and a strong one at 332 nm. At concentrations similar to that present in the enzyme, the product of interaction of free PLP with D-cycloserine had negligible CD. It is thus possible that the decrease in CD intensity at 432 nm over a 4-h period in the reation of D-cycloserine with the enzyme is probably due to the formation of a D-cycloserine–PLP complex. In view of the very low CD

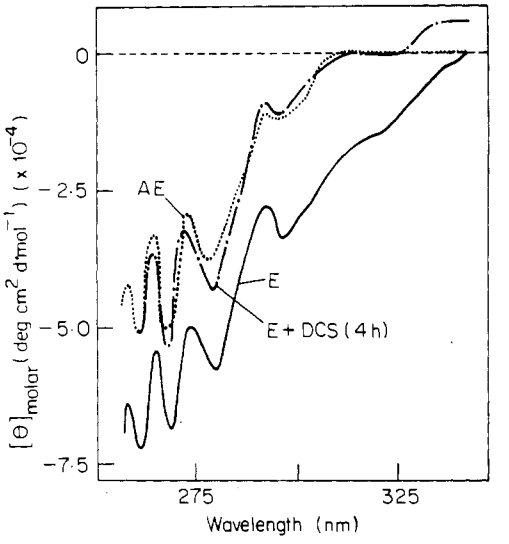

FIGURE 7: Near-UV CD spectra of the holoenzyme (1.5 mg/mL; E), of the holoenzyme (1.5 mg/mL) after incubation with D-cycloserine for 4 h (E + DCS), and of the apoenzyme produced by treatment with L-cysteine (AE; see Methods).

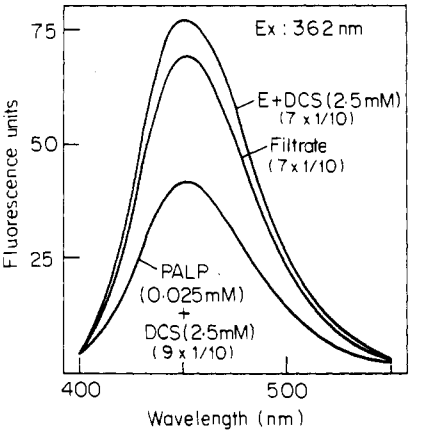

FIGURE 8: Fluorescence emission spectra of the holoenzyme (1.5 mg/mL) upon incubation with D-cycloserine (2.5 mM) for 4 h (E + DCS), of the filtrate obtained by passing the D-cycloserine-treated enzyme through a Whatman glass microfiber filter GF/C (filtrate), and of a solution of PLP (0.025 mM) plus D-cycloserine (2.5 mM) (PALP + DCS). The wavelength of excitation was 362 nm. The numbers in parentheses indicate the instrument settings of selector control and sensitivity control.

intensity of this complex, it was not possible to obtain the characteristic CD spectrum of the PLP–D-cycloserine Schiff's base complex in enzyme solutions.

Visible Fluorescence Spectra. The holoenzyme as well as D-cycloserine had no detectable visible fluorescence. However, the enyzme incubated with 2.5 mM D-cycloserine for 4 h showed a fluorescence spectrum emission with a maximum at 450–455 nm (Figure 8) when excited at 362 nm. When the D-cycloserine-treated enzyme was passed through a glass fiber filter that retained the protein, almost all the dissociable product was recovered in the filtrate as evidenced by the fluorescence spectrum (Figure 8). A similar fluorescence was also obtained when D-cycloserine (2.5 mM) was added to free PLP (0.025 mM) (Figure 8). PMP (0.05 mM) showed no detectable fluorescence at 450 nm when excited at 362 nm.

Discussion

On the basis of the experimental results described above, we propose the following minimal kinetic scheme (Figure 9) for the interaction of D-cycloserine with sheep liver serine hydroxymethyltransferase.

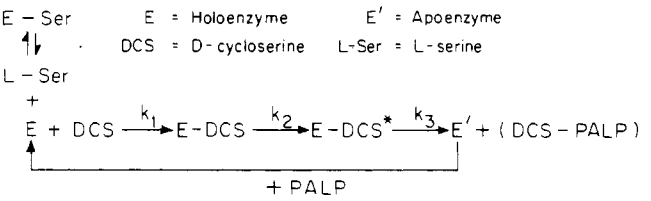

FIGURE 9: Minimal kinetic scheme for the interaction of D-cycloserine with sheep liver serine hydroxymethyltransferase.

D-Cycloserine initially binds rapidly and irreversibly to the enzyme to form an enzyme–D-cycloserine complex (E–DCS) indicated by the disappearance of the enzyme absorbance peak at 425 nm (Figure 2) and by a sudden decrease in the CD intensity at 432 nm (Figure 6). The rate of formation of the E–DCS complex (k_1) is very rapid and is not amenable for measurement by conventional spectroscopic methods. However, by monitoring the subsequent first-order change in enzyme activity (0.5–10 min) and extrapolating to zero time of reaction, we could estimate that this enzyme form might possess a residual enzyme activity of about 27% (Figure 4). The first step can be reversed neither by dilution of the reaction mixture so as to reduce the concentration of the free D-cycloserine nor by the addition of excess substrate, L-serine. Preincubation of the enzyme with an excess amount of L-serine prior to the addition of D-cycloserine, however, slightly lowered the rate of E–DCS formation although the reaction was still irreversible. The protection afforded by preincubating the enzyme with L-serine would be expected if D-cycloserine can only bind to the free enzyme and not to the E–Ser complex.

Karpeiskii et al. (1964) reported the isolation of a stable complex of D-cycloserine with aspartate-glutamate aminotransferase (EC 2.6.1.1), which had 20% of the activity of the native enzyme. This complex had an absorbance maximum at 335 nm, which was attributed to cleavage of the labile carboxamate bond of D-cycloserine and the covalent binding of the resulting reactive acyl group to the carboxyl of an amino acid at the active site. Such a situation does not appear to be operative in the case of serine hydroxymethyltransferase as the apoenzyme prepared by D-cycloserine treatment could be fully reconstituted (Figure 5) and the reconstituted enzyme was fully active.

Accompanying the disappearance of 425-nm peak of the native enzyme is the appearance of a shoulder at about 330 nm (Figure 2). An absorbance peak in the region of 340 nm has been attributed to a transient tetrahedral adduct in the process of formation of a Schiff's base of amino acids with enzyme-bound PLP (O'Leary, 1971). It may be pertinent to mention that a stable tetrahedral adduct has been detected only in the case of serine hydroxymethyltransferase (Schirch, 1982), although such an intermediate has been invoked as a general feature of all PLP-dependent enzymes (Braunstein, 1973). The E–DCS complex is unstable and loses almost all the activity (>95%) in about 30 min (Figure 4). Accompanying this change is a slow decrease in absorbance at 425 nm (Figure 3) and also a slight increase in the CD band intensity at 432 nm (Figure 6). The transformation of the E–DCS complex to the E–DCS* form, which is inactive, occurs at a first-order rate (k_2) of 5.4×10^{-3} s^{-1} (inset of Figure 4). Subsequent to the formation of the E–DCS* complex, there is a slow but significant change in the positive CD band intensity at 432 nm, which reaches nearly zero value in about 4 h (Figure 6). This CD change occurs at a first-order rate (k_3) of 1.4×10^{-4} s^{-1} (inset of Figure 6). The CD band intensity at 432 nm of the E–DCS* complex was obtained by extrapolation of this rate and was found to be about 80% of the intensity of the native enzyme. We should note that since the different steps in the sequence are well separated in time, an extrapolation of this kind would give a good approximation of the CD value of the intermediate species. This final step in the sequence results in an almost complete dissociation of a PLP–D-cycloserine Schiff's base complex from the enzyme active site, resulting in the formation of apoenzyme (Figure 7). The apoenzyme can be reconstituted with PLP to recover the spectral properties and also complete activity of the holoenzyme. The PLP–D-cycloserine complex has very negligible CD at 432 nm as indicated by the CD spectrum of a mixture of D-cycloserine and free PLP. The formation of a PLP–D-cycloserine complex at the end of 4 h was shown by the characteristic fluorescence at 450 nm (Figure 8) of the PLP–D-cycloserine Schiff base (Churchich, 1967). (We could not, however, monitor the release of the PLP–D-cycloserine complex by measuring the fluorescence intenstiy at 450 nm as the intermediate enzyme–D-cycloserine complexes had overlapping fluorescence spectra.) Further, the final PLP–D-cycloserine complex formed at the end of the reaction of D-cycloserine with the enzyme (~4 h) had the same absorbance peak (335 nm) as the product of the interaction of D-cycloserine with free PLP. The reaction of free PLP with D-cycloserine to form a Schiff's base complex proceeds to near completion over a period of time in the presence of a large excess (10^{-2} M) of D-cycloserine, and the reaction is favored at acid pH values (~4) (Churchich, 1967). The final product of the interaction of D-cycloserine with the enzyme-bound PLP is the same as in a nonenzymatic system, and thus the enzyme active site might be viewed as providing a favorable environment for the reaction.

The interaction of D-cycloserine with serine hydroxymethyltransferase differs significantly from its interaction with other PLP–enzymes such as aspartate-glutamate aminotransferase and cystathionase (EC 4.2.1.15) in that the initial phase of this interaction is extremely rapid and irreversible. In the case of both aspartate-glutamate aminotransferase (Khomutov et al., 1963; Karpeiskii et al., 1963, 1964) and cystathionase (Brown et al., 1969; Churchich & Beiler, 1971), the initial phase of the reaction of D-cycloserine is a transaldimation step, which can be reversed by substrate. D-Cycloserine thus reacts with serine hydroxymethyltransferase with a much greater avidity than with other PLP–enzymes. The obvious factors that would be expected to contribute to the affinity of D-cycloserine for PLP–enzymes are (i) the lack of a carboxyl group that would lead to decreased basicity of the α-amino group so as to increase its reactivity and (ii) the lack of conformational freedom in this molecule. It is possible that the active site of serine hydroxymethyltransferase recognizes additional structural features of D-cycloserine that contribute to the firmness of its binding. The subsequent expulsion of the PLP–D-cycloserine Schiff base complex from the enzyme active site could be due to a conformational change at the active site. There is considerable evidence for the conformational mobility at the active site of serine hydroxymethyltransferase consequent to the initial attack by the amino group of glycine or serine. This interaction throws open a site for the binding of the carboxylate group of the amino acid, ultimately leading to the formation of a Schiff's base complex of the amino acid with enzyme-bound PLP (Schirch & Diller, 1971; Cheng & Haslam, 1972; Liu & Haslam, 1973; Akhtar et al., 1975). The Schiff base complexes of PLP with the substrate amino acids, however, remain bound at the active site. It is suggested that binding of the Schiff base complexes of substrate amino acids to the active site is aided by the β-hydroxyl group of the amino acid (Ulevitch & Kallen, 1977a;

Ching & Kallen, 1979). The role of the groups on PLP, which help to anchor the Schiff base complex, has also been investigated (Schirch & Schnackerz, 1978). Since D-cycloserine lacks free carboxyl and hydroxyl groups, our observation that the D-cycloserine–PLP complex dissociates almost completely from the enzyme suggests that the hydroxyl and the carboxyl groups of the substrate amino acids are the major anchoring groups at the active site and not the groups on PLP. The slowness of the dissociation, however, could be due to weak charge interactions at the active site. Further, our observation that D-cycloserine is not cleaved by the enzyme is consistent with the view that complete alkoxide formation at the β-hydroxyl group of a substrate amino acid is required prior to C_α–C_β bond cleavage (Liu & Haslam, 1973; Ulevitch & Kallen, 1977b).

Several PLP-dependent enzymes form complexes with amino acids that exhibit absorbance maxima in the region of 500 nm (Snell & Di Mari, 1970). These intermediates have been ascribed to quinone-like structures formed due to the labilization of an α-proton of the amino acid. In serine hydroxymethyltransferase, similar carbanion or quinonoid intermediates have been observed in the enzyme complexes with glycine and D-alanine (Schirch & Jenkins, 1964a,b; Jordan & Akhtar, 1970; Cheng & Haslam, 1972) especially at high pH. The labilization of the α-proton of the amino acid has been suggested to proceed via a charge-relay type of mechanism involving an amino acid residue at the active site (Ulevitch & Kallen, 1977b). Although D-cycloserine forms a complex with cystathionase that exhibits an absorption maximum at 495 nm (Brown et al., 1969), we failed to observe a similar species in its interaction with serine hydroxymethyltransferase, even at pH 8.5. Since D-cycloserine forms a quinonoid intermediate with cystathionase but not with serine hydroxymethyltransferase, we might conclude that the inhibitor is not properly oriented in the active site of the latter enzyme with respect to the general base that causes α-proton labilization. The free carboxyl groups of D-alanine and glycine are thus perhaps responsible for their proper orientation for the α-proton removal in the active site of serine hydroxymethyltransferase.

A recent paper (Wang et al., 1981) suggests that PMP might be the product of the interaction of D-cycloserine with sheep liver serine hydroxymethyltransferase due to a transamination reaction at the active site of the enzyme similar to the reaction of D-alanine (Schirch & Jenkins, 1964b). We observe that (i) the product of the interaction released into solution at the end of 4 h has the characteristic absorbance maximum (335 nm) and fluorescence emission maximum (450 nm) of a D-cycloserine–PLP Schiff's base complex while PMP, at the same pH, has an absorbance maximum at 325 nm and very negligible fluorescence at 450 nm even at a higher concentration (0.05 mM) and that (ii) the transamination reaction with D-alanine involves a quinonoid intermediate absorbing at 505 nm (Schirch & Jenkins, 1964b) while there is no such intermediate in the reaction with D-cycloserine even at higher pH values.

The very rapid and irreversible initial step of the reaction of D-cycloserine with serine hydroxymethyltransferase is perhaps a critical step in the selective inhibition of this enzyme by the drug both in vivo and in vitro. Since D-cycloserine is a rigid cyclic analogue of both serine and D-alanine, which undergo dealdolization and transamination, respectively, at the enzyme active site, and since the intermediates in its reaction with the enzyme occur in discrete steps of an irreversible reaction sequence, this compound could serve as a useful tool in understanding the properties of the active site of this enzyme.

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Kinetics and Energetics of Intramolecular Electron Transfer in Yeast Cytochrome *c* Peroxidase†

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ABSTRACT: The oxidation of ferric cytochrome *c* peroxidase by hydrogen peroxide yields a product, compound ES [Yonetani, T., Schleyer, H., Chance, B., & Ehrenberg, A. (1967) in *Hemes and Hemoproteins* (Chance, B., Estabrook, R. W., & Yonetani, T., Eds.) p 293, Academic Press, New York], containing an oxyferryl heme and a protein free radical [Dolphin, D., Forman, A., Borg, D. C., Fajer, J., & Felton, R. H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 614–618]. The same oxidant takes the ferrous form of the enzyme to a stable Fe(IV) peroxidase [Ho, P. S., Hoffman, B. M., Kang, C. H., & Margoliash, E. (1983) *J. Biol. Chem. 258*, 4356–4363]. It is 1 equiv more highly oxidized than the ferric protein, contains the oxyferryl heme, but leaves the radical site unoxidized. Addition of sodium fluoride to Fe(IV) peroxidase gives a product with an optical spectrum similar to that of the fluoride complex of the ferric enzyme. However, reductive titration and electron paramagnetic resonance (EPR) data demonstrate that the oxidizing equivalent has not been lost but rather transferred to the radical site. The EPR spectrum for the radical species in the presence of Fe(III) heme is identical with that of compound ES, indicating that the unusual characteristics of the radical EPR signal do not result from coupling to the heme site. By stopped-flow measurements, the oxidizing equivalent transfer process between heme and radical site is first order, with a rate constant of 0.115 s^{-1} at room temperature, which is independent of either ligand or protein concentration. This is slow relative to fluoride binding to the ferric enzyme, indicating that the rate-limiting step is the intramolecular transfer of oxidizing equivalents. Arrhenius plots of the observed rate constants for the Fe(IV) peroxidase and the Fe(III) enzyme yield some of the thermodynamic parameters that describe intermediates in the reaction pathway.

To fully understand the catalytic mechanism for cytochrome *c* peroxidase, one must be able to study both the inter- and the intramolecular electron-transfer reactions involved and their contributions to the overall mechanism. Recently (Ho et al., 1983), we developed a procedure for the preparation in nearly pure form of a cytochrome *c* peroxidase intermediate that is oxidized by 1 equiv above the resting ferric enzyme. This intermediate proved to be the oxyferryl peroxidase not containing the protein free radical normally associated with the fully oxidized hydrogen peroxide product, compound ES (Dolphin et al., 1971). The properties of the Fe(IV) enzyme (denoted as Fe^{IV} R), in conjunction with previous results from reductive titrations (Coulson et al., 1971) and transient-state kinetics for the reduction of compound ES (Jordi & Erman, 1974), allowed the development of a model of the oxidation and subsequent reduction pathways followed by cytochrome *c* peroxidase (Figure 1) (Ho et al., 1983). A novel aspect of this mechanism is the prediction that the conformation of the protein radical site determines its reduction potential. It was proposed that the stable form of the unoxidized site (the R conformer) has a high potential and can be oxidized only by a higher potential heme than that of Fe(IV) peroxidase, so that a significant flow of oxidizing equivalents between the heme and radical sites normally occurs only (1) during the hydrogen peroxide oxidation of the ferric enzyme and (2) within the initial transient intermediate formed upon the one-electron reduction of this oxidized product.

Earlier, Coulson et al. (1971) had reported that the addition of fluoride to a sample of compound ES previously reduced by 1 equiv caused an increase in the radical and the Fe(III) populations. This was interpreted as a shift in the equilibrium of the mixture of intermediates at 1 oxidizing equiv above native in favor of the ferric form, as a result of ligation of the ferric heme iron by the anion. In terms of our proposed mechanism, fluoride binding would serve to increase the reduction potential of the ferryl/ferric redox couple, allowing

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