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Mechanism of Action of D-Serine Dehydratase. Identification of a Transient Intermediate†

Klaus D. Schnackerz,* Jürgen H. Ehrlich, Walter Giesemann, and Thomas A. Reed§

ABSTRACT: Static absorbance measurements of D-serine dehydratase from *Escherichia coli* taken at 2 °C show that during the steady-state course of D-serine conversion the absorption maximum of the Schiff base of the cofactor pyridoxal 5′-phosphate (pyridoxal-P) is shifted from 415 to 442 nm. Furthermore, the progress curve of intermediates was monitored by stopped-flow techniques at wavelengths ranging from 320 to 500 nm. A point by point construction of successive spectra from these stopped-flow traces at various time intervals after the start of reaction resulted in a series of consecutive spectra exhibiting two isobestic points at 353 and 419 nm. The half-time of the absorbance changes occurring at 330 and 455 nm was found to be 6.5 ms, suggesting the observation of a single, enzyme-bound intermediate. The spectral data with substrate and inhibitors provide evidence that the intermediate is the Schiff base of α-aminoacrylate and pyridoxal-P. The proposed assignment is strongly supported by experiments of apodehydratase with transient-state analogues which exhibit a similar absorbance shift on binding to apoenzyme. Moreover, these results suggest that the phosphate group of the substrate–pyridoxal-P complex serves as the main anchoring point during catalysis. A reaction mechanism of the D-serine dehydratase is presented.

The de novo synthesis of D-serine dehydratase is induced upon influx of D-serine into cells of *Escherichia coli*. D-Serine is a competitive antagonist of β-alanine which serves as a substrate in the biosynthesis of pantothenate, a precursor of coenzyme A (Maas & Davis, 1950). D-Serine dehydratase (EC 4.2.1.14) from *E. coli* catalyzes the conversion of D-serine to pyruvate and ammonia (Dupourque et al., 1966; Labow & Robinson, 1966). This enzyme is unique among pyridoxal-P[1] utilizing enzymes studied thus far in that it consists of a single polypeptide chain with one active site (Dowhan & Snell, 1970a). Therefore, the dehydratase is particularly well suited for mechanistic studies without the usual problems of multiple binding sites and subunit interactions.

The absorption spectrum for highly purified D-serine dehydratase exhibits a prominent absorbance maximum at 415 nm, which is due to the cofactor, pyridoxal-P, bound to a lysyl residue of the protein via a Schiff base linkage (Dowhan & Snell, 1970a). The cofactor of this dehydratase is embedded in a polar environment as documented by ^{31}P NMR experiments. Furthermore, by use of this technique it could be shown that the phosphate group of pyridoxal-P has to be in its dianionic form to start catalysis. The formation of a salt bridge of the dianion of the cofactor with most probably an arginine residue of the dehydratase is triggered by the addition of substrate. Hence, this salt bridge now serves at least as one if not the main anchoring point of the cofactor–substrate complex (Schnackerz et al., 1979).

D-Serine dehydratase catalyzes an α,β-elimination reaction, but little is known about the intermediates occurring during catalysis. Other pyridoxal-P dependent enzymes catalyzing the same type of reaction have been studied in some detail. Among those studied were tryptophanase (Snell, 1975) and biodegradative L-threonine deaminase (Rabinowitz et al., 1973; Shizuta et al., 1973), both from *E. coli*. Addition of substrate to L-threonine deaminase caused an intermittent shift of the Schiff base absorbance maximum from 415 to 450 nm, but this transiently occurring absorbance band could not unequivocally be assigned to α-aminoacrylate–pyridoxal-P azomethine (Tokushige et al., 1968; Tokushige & Nakazawa, 1972). In the present study a series of spectroscopic, steady-state, and stopped-flow experiments are described which allow the observation of a single intermediate under the conditions applied. The structure of this intermediate is deduced from its spectral properties in conjunction with experiments of transient-state analogues with D-serine apodehydratase.

Experimental Procedures

Materials. DL-2,3-Diaminopropionate was obtained from Fluka (Buchs, Switzerland). DL-Isoserine was a product of ICN Pharmaceutical, Inc. (Cleveland, OH). DL-*O*-Methylserine was purchased from Sigma Chemical Co. (St. Louis, MO). Pyridoxal hydrochloride, pyridoxal-P, and all other amino acids were obtained from Merck (Darmstadt, West Germany). Sodium pyruvate, NADH, and lactate dehydrogenase were products of Boehringer Mannheim Corp.

† From the Department of Physiological Chemistry, University of Wuerzburg Medical School, Koellikerstr. 2, D-8700 Wuerzburg, Federal Republic of Germany. *Received January 31, 1979.* This research was supported in part by Research Grant Schn 139/4 from the Deutsche Forschungsgemeinschaft. Part of this investigation was submitted by W.G. to the Department of Chemistry of the University of Wuerzburg in partial fulfillment for the degree of Master of Science. A preliminary account of part of this work was presented by the Symposium on Pyridoxal Catalysis sponsored by the U.S.–USSR Academies of Sciences, Leningrad, USSR, Aug 1974.

§ Present address: European Molecular Biology Laboratory, D-6900 Heidelberg, Federal Republic of Germany.

[1] Abbreviations used: NMR, nuclear magnetic resonance; pyridoxal-P, pyridoxal 5′-phosphate.

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^{14}C-Labeled sodium pyruvate and ^{3}H-labeled water were purchased from Amersham-Buchler (Braunschweig, West Germany). Sephadex G-50 was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical reagent grade from Merck.

Enzymes. Crystalline D-serine dehydratase was isolated from *E. coli* K 12 mutant C 6 as described previously (Schiltz & Schnackerz, 1976). Enzyme samples used in these experiments had specific activities ranging from 95 to 120 μmol of D-serine converted to pyruvate per min per mg at pH 7.8 and 25 °C (Schiltz & Schnackerz, 1976). Protein concentrations were determined either spectrophotometrically by using an extinction coefficient of $A_{280}^{1\%}$ of 10.5 (Dowhan & Snell, 1970a) or by the Lowry method (Lowry et al., 1951). D-Serine apodehydratase was prepared by using the resolution procedure of Dowhan & Snell (1970a). The apoenzyme had a residual specific activity of 1.8 μmol of pyruvate formed per min per mg.

Synthesis of Analogues of α-Aminoacrylate–Pyridoxal-P Schiff Base. (1) 4-[2-Methyl-3-hydroxy-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic Acid (I). Pyridoxal-P (1.5 mmol) and sodium pyruvate (6 mmol) were dissolved in 80 mL of 0.5 M KOH, and the solution was stirred for 24 h in the dark at room temperature. The reddish reaction mixture was adjusted to pH 7.0 with 70% perchloric acid and then concentrated by rotary evaporation and separated from solid $KClO_4$. The remaining salt was washed twice with cold water. The combined supernatants were concentrated (2 mL) and applied to a Dowex 50 W-X 4 column (H^+ form; 200–400 mesh; 2 × 50 cm). The reaction products were eluted with 500 mL of distilled water, collecting 8-mL fractions. Aliquots of each fraction were spotted on silica gel thin-layer plates, developed in 1-butanol–acetic acid–water (6:2:2 by volume), and characterized with Gibbs reagent spray (Gibbs, 1927). Fractions 26–53 containing the oxobutenoic acid derivative were collected, concentrated (5 mL), and treated with ethanol until turbidity appeared. The product was recrystallized from ethanol–water. The yield was 240 mg (50% of theory); mp 116 °C (dec). Anal. Calcd for $C_{11}H_{12}O_8NP{\cdot}H_2O$ (M_r 335.2): C, 39.41; H, 4.21; N, 4.18. Found: C, 39.14; H, 4.41; N, 4.69. The spectrum in 100 mM potassium phosphate buffer, pH 7.8, exhibits maxima at 412, 292, and 245 nm, respectively. The radioactively labeled oxobutenoic acid derivative was prepared by mixing 2.35 mg of sodium [1-^{14}C]pyruvate with a specific radioactivity of 11.7 mCi/mmol with 40 mg of cold pyruvate and 10 mg of pyridoxal-P in 5 mL of 0.5 M KOH. The treatment was the same as that described above, except that the reaction product was purified successively on two cation exchange columns. The yield was 6.3 mg (52% of theory).

(2) 4-[2-Methyl-3-hydroxy-5-(hydroxymethyl)-4-pyridinyl]-2-oxo-3-butenoic Acid (II). Pyridoxal hydrochloride (6 mmol) and sodium pyruvate (24 mmol) were dissolved in 100 mL of 0.5 M KOH, and the solution was kept with stirring for 24 h at room temperature in the dark. The resulting dark red solution was treated with perchloric acid and handled as described for analogue I. The reaction products were separated on a Dowex 2-X8 column (acetate form; 200–400 mesh; 2.5 × 35 cm) with 1 L of a linear gradient ranging from 0 to 2 M acetic acid. Fractions (8 mL) were collected and analyzed as described above. Unreacted pyridoxal was eluted in fractions 10–15, whereas the pure analogue II was found in fractions 20–59. These fractions were collected and concentrated to dryness. The crude product was recrystallized from hot water. Feltlike white crystals were obtained in a yield of 500 mg (34% of theory); mp 208–209 °C (dec). Anal. Calcd for $C_{11}H_{11}O_5N$ (M_r 237.2): C, 55.70; H, 4.67; N, 5.91. Found: C, 55.88; H, 4.79; N, 5.98. The spectrum in potassium phosphate buffer, pH 7.8, showed maxima at 312 and 412 nm and a shoulder at 255 nm, respectively.

(3) 4-[2-Methyl-3-hydroxy-5-(phosphooxymethyl)-4-pyridinyl]-2-oximino-3-butenoic Acid (III). I (315 nmol) and hydroxylamine hydrochloride (360 nmol) were dissolved in 20 mL of distilled water and kept in the dark for 15 h with stirring. Concentration of the reaction mixture produced the yellow oxime which can be recrystallized from water–acetone. The yield was 90 mg (82% of theory); mp 167 °C (dec). Anal. Calcd for $C_{11}H_{13}O_8N_2P{\cdot}H_2O$ (M_r 350.23): C, 37.72; H, 4.32; N, 8.00. Found: C, 37.15; H, 4.43; N, 8.04. The spectrum in 100 mM potassium phosphate buffer, pH 7.8, displayed maxima at 246, 302, and 376 nm, respectively.

(4) 4-[2-Methyl-3-hydroxy-5-(hydroxymethyl)-4-pyridinyl]-2-oximino-3-butenoic Acid (IV). II (1.26 mmol) was dissolved in 7 mM NaOH (100 mL) and upon addition of 1.44 mmol of hydroxylamine hydrochloride kept in the dark with stirring for 48 h at room temperature. The concentrated reaction mixture yields 300 mg of oxime (88% of theory), mp 152–153 °C (dec). Anal. Calcd for $C_{11}H_{12}O_5N_2{\cdot}H_2O$ (M_r 270.25): C, 48.89; H, 5.22; N, 10.37. Found: C, 48.47; H, 5.19; N, 10.29.

Reaction of D-Serine Apodehydratase with Analogues. One milliliter each of 91 μM apodehydratase and of an equimolar solution of I and a fivefold excess of II or III, respectively, were placed in different compartments of a tandem cuvette and equilibrated to 10 °C in a thermostated cuvette holder of a Cary Model 15 recording spectrophotometer. After mixing the contents of the two chambers, we recorded the absorbance in the range from 500 to 320 nm at various time intervals. Completion of the reaction was judged by ceasing changes in absorbance. The reaction mixture was then applied to a Sephadex G-50 column (1 × 15 cm), preequilibrated with 100 mM potassium phosphate buffer, pH 7.8, and eluted with the same buffer. Fractions (1.5 mL) were collected, and protein concentrations and radioactivity were determined.

Absorbance Measurements. Static absorbance spectra were taken in 100 mM potassium phosphate buffer, pH 7.8, on a Cary Model 15 recording spectrophotometer thermostated to 2 °C using cuvettes with a 1-cm light path. Reaction of apoenzyme with analogues of α-aminoacrylate–pyridoxal-P Schiff base was followed at 10 °C by using tandem cuvettes with a total light path of 0.874 cm.

Radioactivity Measurements. Radioactivity of ^{14}C-labeled analogues and ^{3}H-labeled compounds was determined in Bray's solution (Bray, 1960) on a Packard Model 3380 liquid scintillation spectrometer. Isotope experiments were corrected for counting efficiency.

Tritium Exchange Experiments. DL-[2-^{3}H]Serine was prepared according to the method described by Greenstein & Winitz (1961). The tritiated DL-serine was purified by chromatography on Dowex 50-X8 (H^+ form; 1 × 10 cm). After elution with 1 M NH_4OH the amino acid was recrystallized from ethanol and had a specific radioactivity of 2949 dpm/μmol. Incubation of DL-[2-^{3}H]serine (60 μmol) in 100 mM potassium phosphate buffer, pH 7.7, with 1.5 units of D-serine dehydratase was allowed for 2 and 4 min at 25 °C, converting approximately 10 and 20% D-serine, respectively. In addition, tritiated DL-serine (30 μmol) reacted in the presence of 15 units of D-serine dehydratase in 15 min to completion. In all cases, the reaction was stopped by adding perchloric acid to a final concentration of 0.228 M. The acidified solutions were neutralized with 2 M potassium bi-

carbonate. Denatured protein and precipitated $KClO_4$ were removed by centrifugation. The pyruvate concentration of the supernatant was measured enzymatically by using lactate dehydrogenase. The major part of the supernatant was transferred to one arm of a modified two-arm Thunberg tube. After evacuation, water was distilled to the other arm precooled to the temperature of liquid nitrogen. The tritium content of the distillate was determined by radioactivity measurements.

The incorporation of tritium from $[^3H]H_2O$ into glycine and D-alanine was measured in the following fashion. The amino acids (200 mM, 0.2 mL) dissolved in 100 mM potassium phosphate buffer, pH 7.8, were incubated with 80 units of D-serine dehydratase and 10 μL of $[^3H]H_2O$ (150 mCi/mL) for 43 h at 25 °C in the dark. Controls without enzyme were treated likewise. The reaction was terminated by heating the samples in boiling water for 5 min. After centrifugation the reaction mixtures were repeatedly taken to dryness in vacuo. The residual products were dissolved in 200 μL of water, spotted on silica gel thin-layer plates, and developed in 1-butanol–acetic acid–water (6:2:2 by volume). The amino acid containing areas were collected and eluted with water. Aliquots were taken to determine the amino acid content by using an amino acid analyzer and the radioactivity. Similar experiments were performed with D-threonine, and the reaction was stopped after it had gone to 60% completion. Unreacted D-threonine was isolated as described for glycine.

Stopped-Flow Measurements. Stopped-flow measurements were performed on two different Gibson-Durrum stopped-flow spectrometers equipped with a 2-cm path length cell. The stop syringes in both instruments were adjusted to display a short portion of the flow mode to simplify identification of the start of the reaction. Traces from one of these spectrometers were recorded on a Nicolet Model 1090 digital oscillograph and transferred via paper tape punch and tape reader to a Wang Model 720 C programmable calculator for processing and printout. Traces from the other instrument were read out via a log linear amplifier to a Nicolet Model 1070 instrument computer and transferred via an 8-bit parallel interface to a Wang Model 2000 computer for processing and printout. For low-temperature studies (6 °C), dry nitrogen was blown onto the cuvette windows until just before the reaction was initiated. The draw tubes leading to and from the cuvette windows retained sufficient dry nitrogen to prevent fogging during the course of the reaction. The instrument dead time was 2.5 ms. Data for the point by point curves were collected on a single enzyme sample (12.5 μM in the cuvette). The instrument was adjusted to a new spectroscopic zero for each wavelength measured, and the readout was taken in optical density units. All stopped-flow measurements were run at least in duplicate. Repeated stopped-flow traces were normally superimposable when inspected visually. In cases of low absorbance changes resulting from high signal to noise ratios, the absorbance values were checked by signal averaging four successive traces. Absorbance values for each time point were averaged for 375 μs on each side of the readout time (15 points at a dwell time of 50 μs/point). The wavelengths of the isobestic points were first estimated visually. For a number of wavelengths on each side of those estimated points, the difference between the average absorbance for the first 5 ms of flow mode and that of the last 5 ms of observation was used to calculate the location of the isobestic points. These absorbance differences plotted as a function of wavelength gave straight lines (420-nm region, five values, coefficient of confidence 0.99; 350-nm region, six values, coefficient of confidence 0.97). The actual isobestic points were obtained by extrapolation to zero absorbance difference. The standard deviation of the absorbance-difference measurements was 0.002 optical density units. The integration of the absorbance–time curves required for demonstration of obligatory intermediates was accomplished either by plotting on millimeter scale graph paper and square counting or by exactly cutting the area under the curve and weighing the cut paper on an analytical balance.

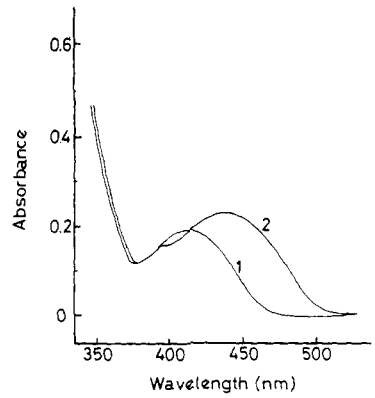


FIGURE 1: Absorption spectra of D-serine dehydratase. Curve 1 represents enzyme alone. Curve 2 illustrates enzyme (32 μM) during reaction with 33 mM D-serine 1.5 min after mixing. The measurements were taken at 2 °C in 100 mM potassium phosphate buffer, pH 7.8. The light path of the cuvette was 1 cm.

Results

Absorbance Changes in the 460-nm Region. The absorption spectrum of D-serine dehydratase shows a maximum at 415 nm (Figure 1, curve 1), typical for one class of pyridoxal-P utilizing enzymes (Snell & DiMari, 1970; Johnson & Metzler, 1970). Variation of the pH in the range from 5.5 to 9.5 does not alter the position of this maximum. If conditions were chosen to prolong the reaction sufficiently to obtain absorption spectra during the steady-state course of D-serine conversion, a maximum at 442 nm (Figure 1, curve 2) is observed. At the end of the reaction the spectrum reverts to that of unreacted enzyme.

These static absorbance measurements give no information on the number of species which may occur as intermediates. The absorbance change could indicate the appearance of a single species but could also connotate the simultaneous concentration change of several species. Therefore, the progress curve of the intermediates was monitored by stopped-flow techniques at a number of wavelengths in the range from 320 to 500 nm at 5 °C. Difficulties in resolving reaction times dictated a lower temperature. A point by point construction of successive spectra of D-serine dehydratase during the reaction process from stopped-flow traces at various time intervals after the start of reaction is illustrated in Figure 2. The series of consecutive spectra exhibit two isobestic points at 353 and 419 nm, suggesting the observation of a single, enzyme-bound intermediate. The difference curve taken from the stopped-flow traces at the end of flow mode and 50 ms after mixing shows a maximum around 470 nm and a trough around 390 nm. A wavelength of 455 nm was chosen to observe the formation of the enzyme-bound intermediate. For simplicity, in the following description and discussion the 442-nm static absorbance maximum and the 470-nm dynamic difference maximum obtained from stopped-flow traces are referred to as the 455-nm intermediate.

Competitive inhibitors for D-serine dehydratase, such as DL-2,3-diaminopropionate, DL-isoserine, DL-*O*-methylserine, L-serine, and glycine, show only slight increases in absorbance

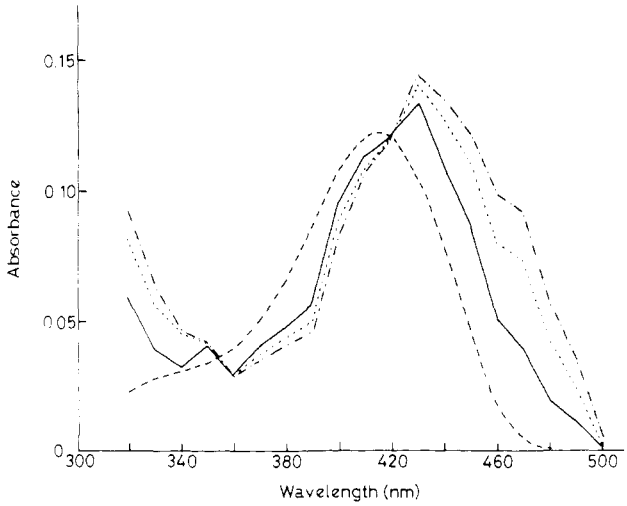


FIGURE 2: Point by point construction of successive spectra of D-serine dehydratase during the reaction process from stopped-flow traces. Reaction conditions in the cuvette: enzyme, 12.5 μM; D-serine, 1.25 mM; temperature, 5 °C. For details see Experimental Procedures. (---) D-Serine dehydratase only; (—) 7, (···) 17, and (-·-) 47 ms after the start of the reaction, respectively.

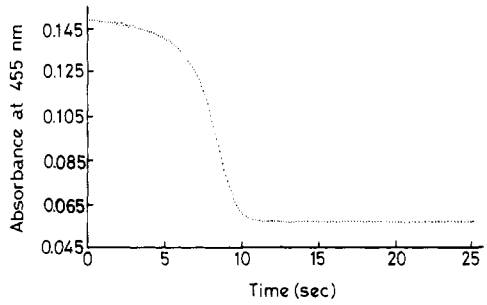


FIGURE 3: Stopped-flow reaction curve of D-serine dehydratase reaction observed at 455 nm. Disappearance of intermediate V at exhaustion of substrate. Reaction conditions: D-serine dehydratase, 25 μM; D-serine, 5 mM; potassium phosphate buffer, 100 mM (pH 7.8); temperature, 6 °C.

at 415 nm but no bathochromic shift at all even when added in large excess. The rapid increase in Schiff base fluorescence emission upon addition of these inhibitors to D-serine dehydratase indicated the formation of enzyme–inhibitor complexes (Ehrlich & Schnackerz, 1973).

To establish the obligatory nature of the 455-nm intermediate during D-serine conversion, we determined the time integral of the 455-nm absorbance ($\int \Delta A_{455}\, dt$), i.e., the integration of the extinction–time curve describing the duration of the 455-nm extinction with time, at various substrate and enzyme concentrations. The necessary reaction curves were obtained from stopped-flow experiments since this technique minimizes mixing dead time errors inherent in such measurements by conventional spectrophotometric methods. A typical reaction curve, depicted in Figure 3, demonstrates the course of the 455-nm absorbance at 6 °C. Since it is known that the intermediate spectrum returns to that of the unreacted enzyme after completion of the reaction, the straight line at the end of the reaction was used as base line for the integration. The plot of the time integral of ΔA_{455} during the entire course of the reaction vs. substrate concentration at a constant enzyme level shows strict linearity; i.e., an increase in substrate concentration produces a proportional increase in the transiently occurring 455-nm intermediate (Figure 4A). At constant substrate concentrations, however, the time integral of ΔA_{455} remains constant with increasing enzyme levels (Figure 4B); i.e., an increase in enzyme concentration produces

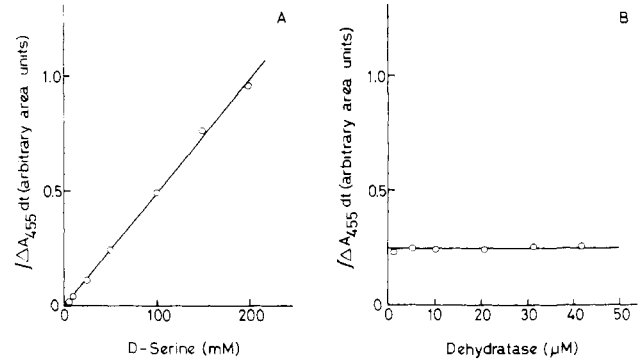


FIGURE 4: (a) Plot of time-integrated ΔA_{455} as a function of substrate concentration. The enzyme concentration was 5 μM. (B) Plot of time-integrated ΔA_{455} as a function of enzyme concentration. The substrate concentration was 50 mM. All other conditions were as stated in the legend of Figure 3.

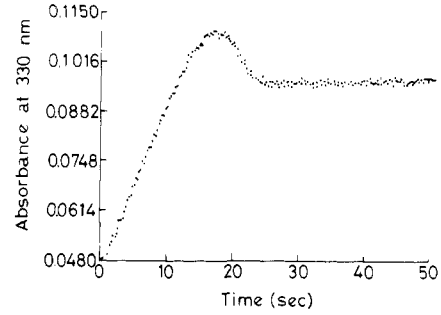
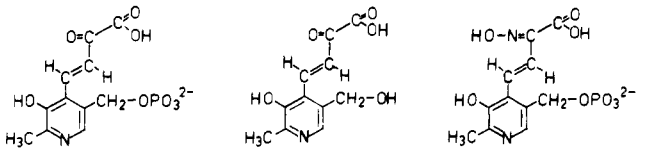


FIGURE 5: Stopped-flow curve of D-serine dehydratase reaction observed at 330 nm. Reaction conditions: dehydratase, 12.5 μM; D-serine, 5 mM. All other conditions were as stated in the legend of Figure 3.

the same amount of transiently occurring 455-nm intermediate in a proportionally shorter time period. These observations indicate that the enzyme species absorbing at 455 nm is an obligatory intermediate in the catalytic mechanism of this dehydratase. At 12.5 mM substrate, the average half-time for the formation of the 455-nm intermediate taken at wavelengths from 430 to 490 nm was 6.5 ± 0.5 ms. The disappearance of the 455-nm absorbance at exhaustion of substrate involves no residual slow change (Figure 3).

Absorbance Changes in the 330-nm Region. The observation of a second isobestic point at 353 nm (Figure 2) prompted a stopped-flow study on D-serine dehydratase during turnover of substrate in the 330-nm region. The determination of the intermediate at this wavelength is, however, complicated by the fact that the reaction product, pyruvate, is known to absorb in this region. Figure 5 demonstrates the rise in 330-nm absorbance with an overshoot which reverts to a constant level after substrate exhaustion, the latter indicating the total amount of pyruvate formed. On the other hand, the overshoot during turnover of substrate disclosed the transient appearance of an intermediate. At greater time resolution, absorbance changes at 330 nm with two different reaction rates could be distinguished. For the faster reaction a half-time of approximately 6.5 ms was calculated from stopped-flow traces at 12.5 mM substrate. The second, slower reaction undoubtedly indicated the formation of pyruvate (43 μmol of pyruvate per min per mg of protein). The similar half-times observed at 330 and 455 nm suggest that the overshoot reflects the appearance and disappearance of the 455-nm intermediate and not the formation of another spectral species.

Synthesis of Transient-State Analogues of α-Aminoacrylate–Pyridoxal-P Schiff Base. The α-aminoacrylate–pyridoxal-P azomethine was proposed as a key intermediate



I II III

FIGURE 6: Structures of transient-state analogues of α-aminoacrylate–pyridoxal-P Schiff base.

in α,β-elimination reactions of amino acids catalyzed by pyridoxal-P dependent enzymes [for a review, see Davis & Metzler (1972)]. Spectral bands at 460–470 nm found for certain enzymatic α,β-elimination reactions of amino acids were assigned to either the α-aminoacrylate–pyridoxal-P Schiff base or the quinonoid intermediate occurring after proton abstraction from the α carbon of the amino acid–pyridoxal-P azomethine (Tokushige et al., 1968; Rabinowitz et al., 1973; Kredich et al., 1969; Morino & Snell, 1967). The spectral properties of the α-aminoacrylate–pyridoxal-P azomethine, however, have never directly been established due to the instability of this compound. Therefore, a number of transient-state analogues were synthesized by aldol condensation of pyridoxal-P or pyridoxal with pyruvate, followed by water abstraction in alkaline solution. In these analogues the C=N bond was substituted by a C=C bond, and the C=C bond of the acrylate moiety was substituted by a carbonyl function or an oxime group (Figure 6).

Reaction of Transient-State Analogues with D-*Serine Apodehydratase.* The absorbance maximum of the phosphorylated analogue I at 412 nm is shifted to 460 nm upon incubation with an equimolar amount of D-serine apodehydratase at 10 °C, and concomitantly, a shoulder centered around 330 nm appeared, as shown in Figure 7. During the formation of the apodehydratase–analogue complex which is completed within 45 min at 10 °C, two isobestic points at 440 and 350 nm have been observed. It should be noted that the apodehydratase solution has to be saturated with nitrogen and be free of any sulfhydryl reagent used as antioxidant since the latter would instantaneously react with the analogue in a Michael condensation reaction. The complex once formed cannot be resolved by gel filtration on Sephadex G-50. Incubation of apodehydratase with a fourfold excess of ^{14}C-labeled analogue I for 15 min at room temperature, separation of the complex from excess reagent on Sephadex G-50, and determination of protein concentration and radioactivity of the separated complex showed that analogue I binds to apodehydratase in a 1:1 ratio. The complex is inactive as was to be expected. Reconstitution experiments with equimolar amounts of pyridoxal-P restored half of the activity in 2 h when compared with the reconstitution capacity of the apodehydratase itself.

In contrast, the nonphosphorylated analogue II, when incubated even in fivefold excess with D-serine apodehydratase for 2 h at 10 °C, showed no change in absorbance intensity and no spectral shift. The components could readily be separated by gel filtration on Sephadex G-50, indicating that analogue II binds very weakly if at all to apoenzyme. On the other hand, the phosphorylated analogue III which represents the oxime derivative of I changes the location of its maximum from 375 to 430 nm when incubated with apodehydratase. The binding of analogue III to apoenzyme even at a fourfold excess of the analogue is much slower than that observed for analogue I. Nevertheless, in an experiment similar to that described for analogue I, an analogue III to apoenzyme ratio of 1.2 was found.

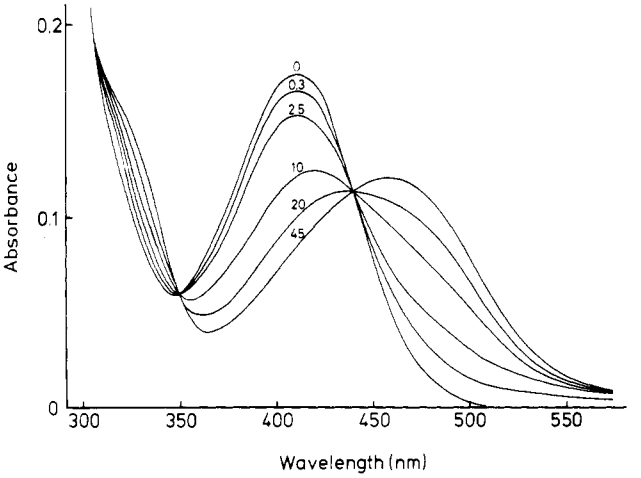


FIGURE 7: Reaction of D-serine apodehydratase with transient-state analogue I. Reaction conditions in the tandem cuvette prior to mixing: apodehydratase, 91 μM; analogue I, 91 μM; temperature, 10 °C; potassium phosphate buffer, 100 mM (pH 7.8). The light path of the tandem cuvette was 0.874 cm. The numbers on the curves indicate the time after mixing when the scan was performed.

Tritium Exchange. Two types of tritium exchange experiments were performed. First, competitive inhibitors, such as glycine and D-alanine, were incubated for 43 h at pH 7.8 with enzyme in the presence of $[^3H]H_2O$. Tritium incorporations of 14.8 and 0.3% were found for reisolated, purified glycine and D-alanine, respectively. Even though the incorporation of tritium label into D-alanine appears to be insignificant, it is nevertheless at least 20 times higher than that of the control. In contrast, in a similar experiment with D-threonine, a poor substrate of D-serine dehydratase, no tritium label was detected in the reisolated amino acid when the reaction was terminated after 60% completion. Second, DL-[2-^3H]serine was incubated at pH 7.7 with D-serine dehydratase, and the reaction was discontinued after various degrees of substrate turnover. The water was separated from all other reactants by distillation. After 8.9, 23.7, and 100% D-serine was converted, the ratio of tritium label in the solvent to the product formed was determined to be 3071, 2568, and 3010 dpm/μmol of pyruvate, respectively. The stock solution of tritiated DL-serine used contained 2949 dpm/μmol of D-serine. These results indicate that no isotopic effect occurred. Thus, the removal of the α hydrogen of D-serine is not rate limiting.

Discussion

The accepted mechanism for α,β-elimination reactions of amino acids catalyzed by pyridoxal-P containing enzymes is presented in Figure 8 (Morino & Snell, 1967; Davis & Metzler, 1972; Rabinowitz et al., 1973). This mechanism was first suggested for tryptophanase by Morino & Snell (1967). Addition of substrate, D-serine, to the lysyl-pyridoxal-P Schiff base forms an aldamine adduct II which is rearranged to the first transaldimination product III upon release of the ϵ-amino group of the enzyme-originated lysine. Then the hydrogen from the α carbon of D-serine is abstracted as a proton. The resulting carbanion or the quinonoid intermediate IV, two resonance structures of the same ground state, can generate the α-aminoacrylate–pyridoxal-P azomethine intermediate V after release of the hydroxyl ion from the β carbon of D-serine. Attack of the ϵ-amino group of the enzyme-lysyl residue at the 4′ carbon of the pyridine moiety of the intermediate V facilitates the addition of a proton at the β carbon of the amino acid forming another aldamine-type intermediate VI which is finally rearranged in a second transaldimination step to form

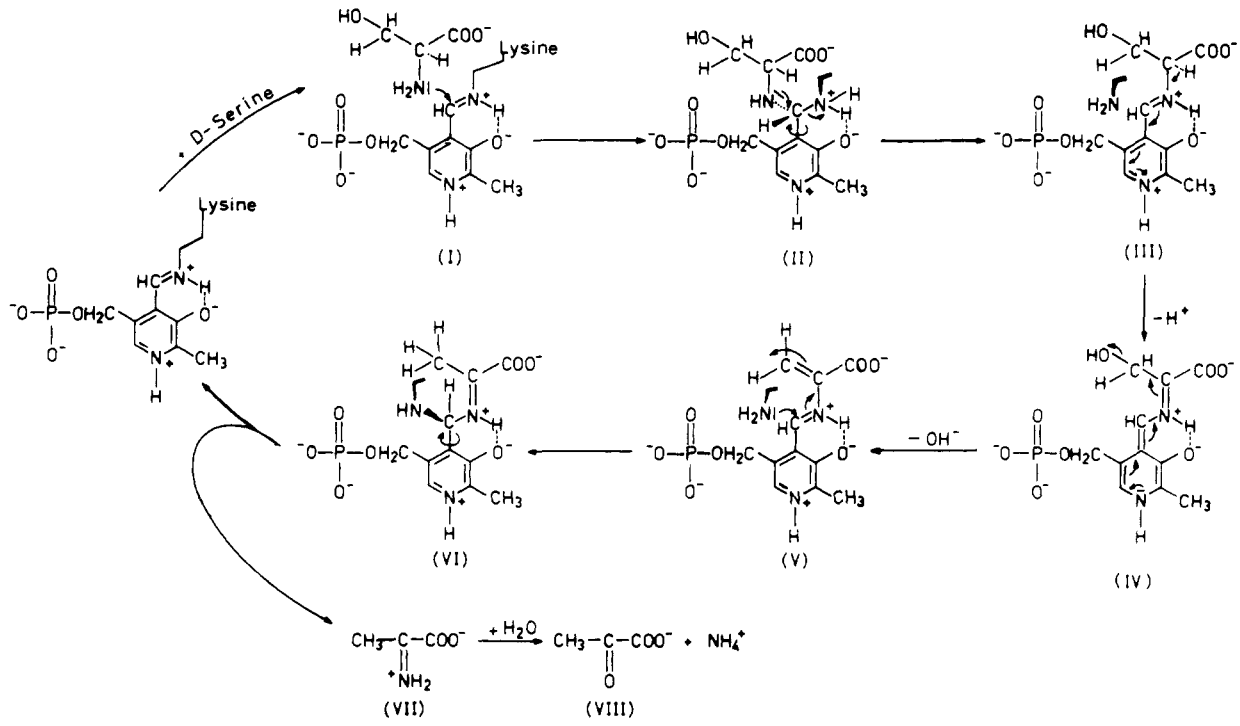


FIGURE 8: Detailed catalytic reaction mechanism of D-serine dehydratase.

the original lysyl-pyridoxal-P Schiff base and iminopropionic acid. The iminopropionate is then hydrolyzed to pyruvate and ammonium ions. The final conversion of intermediate V to pyruvate occurs in D-serine dehydratase stereospecifically as documented by Huang (1972), suggesting that product formation takes place prior to its release from the enzyme.

The steady-state reaction (Figure 1) with maximum at 442 nm indicates the formation of transient reaction intermediates but gives no conclusive answer to the number of intermediates contributing to the maximum or the development of other intermediates prior to steady state. The kinetic reaction curves exhibit two isobestic points at 419 and 353 nm. The occurrence of two isobestic points under these conditions shows that the transient responsible for a particular developing peak consists of only one species. If, for example, the maxima at longer wavelength in Figure 2 were a mixture of two or more species, the ratios of these components would be expected to vary during the development of steady state. In this case, the isobestic point at 419 nm would shift as shown in studies by Ray (1956). It is therefore fully justified to use the 455-nm stopped-flow curves to monitor the development of the single intermediate.

The relation between the time integral of ΔA_{455} and the D-serine concentration at constant enzyme level and enzyme concentration at constant substrate level, respectively, provides good evidence for the obligatory nature of this intermediate in the catalytic reaction mechanism of D-serine dehydratase.

The 455-nm intermediate is suggested to represent the Schiff base of pyridoxal-P and α-aminoacrylate. The dominance of the 455-nm intermediate with a half-time of 6.5 ms and the relatively slow formation of pyruvate show that the rate-limiting step of the overall reaction occurs after the formation of the azomethine V. The identity of the 455-nm intermediate is based on spectral data both with competitive inhibitors and with substrate itself. D-Serine dehydratase binds competitive inhibitors as indicated by the slight increase in 415-nm absorbance, but no bathochromic shift can be observed. These results suggest that only the formation of the respective transaldimination product III is spectroscopically detectable. Incubation of glycine and D-alanine with dehydratase in tritiated water for 43 h has shown only partial incorporation of tritium into these amino acids. No tritium label was found in reisolated D-threonine when the reaction was stopped after it had gone to 60% completion. Therefore, it seems that the corresponding intermediate IV can be generated from these inhibitors, but their respective steady-state concentrations are far too small to be observed by spectroscopic techniques. Spectral evidence for the α-aminoacrylate–pyridoxal-P Schiff base is further supported by the fact that, for the addition of one double bond to the conjugated system of the pyridoxal-P azomethine with D-serine, a bathochromic shift of the absorption maximum by 15–50 nm would be expected (Dryer, 1965). Although the size of the shift clearly depends on the structure of the molecule involved and the nature of the surroundings, the observed bathochromic shift is well in the range of theoretical predictions for a Schiff base of α-aminoacrylate and pyridoxal-P. The quinonoid derivative IV is not likely to be the 455-nm intermediate since, in detailed studies on pyridoxal-P dependent enzymes which accumulate such quinonoid ketamine intermediates, absorption maxima in the 490–530-nm region invariably have been found (Jenkins, 1964; Schirch & Jenkins, 1964; Morino & Snell, 1967). This assignment is unambiguous because α,β-unsaturated intermediates, such as α-aminoacrylate–pyridoxal-P Schiff base, do not participate in the catalytic mechanism of these enzymes, except in tryptophanase. For the latter enzyme, however, the β-elimination step has firmly been established as the rate-limiting step (Morino & Snell, 1967).

At first sight the absorbance increase in the 330-nm region seemed to indicate the formation of the product, pyruvate, which is known to absorb at this wavelength. Critical evaluation of the kinetic measurements at 330 nm at various time scales reveals not only the slow formation of pyruvate but also the transient appearance of another spectral species clearly documented by the time-dependent absorbance increase (Figure 5). A half-time of about 6.5 ms can be calculated for the faster reaction. The absorbance increase of the intermediate at 330 nm was to be expected from the point by point

kinetic spectrum (Figure 2) in which the two isobestic points suggested the occurrence of a single transient intermediate. The similar rate constants for the absorbance increase at 455 and 330 nm support the evidence that the absorbance changes at both wavelengths are produced by a single intermediate, α-aminoacrylate–pyridoxal-P Schiff base. Binding of *O*-acetyl-L-serine to *O*-acetylserine sulfhydrylase gives rise to absorbance changes both at 470 and at 330 nm due to the formation of the same intermediate generated upon release of acetate from *O*-acetyl-L-serine (Cook & Wedding, 1976). It should be mentioned that absorption bands in the 330-nm region have also been observed for aldamine structures (Buell & Hansen, 1960; Schuster & Winkler, 1970; Jo et al., 1977; Feldmann & Helmreich, 1976), but aldamines, like intermediates II and VI, do not absorb at 470 nm.

Additional evidence to confirm the structure of the single intermediate observed was provided by spectral data of the transient-state analogues I and III when attached to D-serine apodehydratase. Incubation of phosphorylated transient-state analogue I with apoenzyme produced a 1:1 complex. The absorbance maximum of analogue I concomitantly shifts from 412 to 460 nm, indicating a specifically altered arrangement of its conjugated π-electron system when fixed in the active site of the dehydratase. It should be noted that upon formation of the complex an absorbance increase around 330 nm was observed, providing additional evidence for a single intermediate producing absorbance changes at two wavelengths. The complex formed was so strong that is could not be separated into its individual components by gel filtration. Analogue I was, however, not irreversibly attached to apoenzyme since half of the enzymatic activity could be regained upon incubation with pyridoxal-P. The reason why fully reconstituted enzyme was never obtained from the complex might be sought in the extreme sensitivity of the apoenzyme toward oxygen (Dowhan & Snell, 1970a). The sulfhydryl reagent normally used to protect dehydratase-originated cysteine side chains had to be omitted due to their prompt reaction with analogue I. On the other hand, from experiments with nonphosphorylated analogue II and apoenzyme, it has to be concluded that this analogue cannot form a complex with apoenzyme most probably because it is lacking the very important phosphate group. This finding is in accordance with results of binding studies on nonphosphorylated pyridoxal-P analogues to D-serine apodehydratase (Dowhan & Snell, 1970b). In conclusion, ^{31}P NMR studies with the competitive inhibitor isoserine have shown that the formation of the first transaldimination product lacking the 470-nm absorbance maximum is coupled with the formation of a salt bridge between the phosphate group of intermediate III and most probably an arginine residue (Schnackerz et al., 1979). Furthermore, the experiments with the transient-state analogues I–III and apodehydratase indicate that the complex is only formed in the case of analogues I and III. These results can be interpreted in that the phosphate group of intermediate V is still necessary for the attachment to apoenzyme. Hence, the phosphate group of the substrate–pyridoxal-P complex serves as the main anchoring point during catalysis.

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