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Reaction Mechanism of *Escherichia coli* Cystathionine γ-Synthase: Direct Evidence for a Pyridoxamine Derivative of Vinylglyoxylate as a Key Intermediate in Pyridoxal Phosphate Dependent γ-Elimination and γ-Replacement Reactions†

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ABSTRACT: Cystathionine γ-synthase catalyzes a pyridoxal phosphate dependent synthesis of cystathionine from *O*-succinyl-L-homoserine (OSHS) and L-cysteine via a γ-replacement reaction. In the absence of L-cysteine, OSHS undergoes an enzyme-catalyzed, γ-elimination reaction to form succinate, α-ketobutyrate, and ammonia. Since elimination of the γ-substituent is necessary for both reactions, it is reasonable to assume that the replacement and elimination reaction pathways diverge from a common intermediate. Previously, this partitioning intermediate has been assigned to a highly conjugated α-iminovinylglycine quininoid (Johnston et al., 1979a). The experiments reported herein support an alternative assignment for the partitioning intermediate. We have examined the γ-replacement and γ-elimination reactions of cystathionine γ-synthase via rapid-scanning stopped-flow and single-wavelength stopped-flow UV-visible spectroscopy. The γ-elimination reaction is characterized by a rapid decrease in the amplitude of the enzyme internal aldimine spectral band at 422 nm with a concomitant appearance of a new species which absorbs in the 300-nm region. A 485-nm species subsequently accumulates in a much slower relaxation. The γ-replacement reaction shows a red shift of the 422-nm peak to 425 nm which occurs in the experiment dead time (~3 ms). This relaxation is followed by a decrease in absorbance at 425 nm that is tightly coupled to the appearance of a species which absorbs in the 300-nm region. Reaction of the substrate analogues L-alanine and L-allylglycine with cystathionine γ-synthase results in bleaching of the 422-nm absorbance and the appearance of a 300-nm species. In the absence of L-cysteine, L-allylglycine undergoes facile proton exchange; in the presence of L-cysteine, L-allylglycine undergoes a γ-replacement reaction to form a new amino acid, γ-methylcystathionine. No long-wavelength-absorbing species accumulate during either of these reactions. These results establish that the partitioning intermediate is an α-imino β,γ-unsaturated pyridoxamine derivative with $\lambda_{max} \simeq 300$ nm and that the 485-nm species which accumulates in the elimination reaction is not on the replacement pathway.

Cystathionine γ-synthase is an important pyridoxal phosphate (PLP)[1] dependent enzyme involved in methionine biosynthesis. It is the only known enzyme with a physiological function to catalyze a replacement reaction at the γ-carbon of an amino acid. The enzyme from *Escherichia coli* catalyzes the production of L,L-cystathionine from *O*-succinyl-L-homoserine (OSHS) and L-cysteine (L-Cys) (eq 1). In the absence of L-Cys, the enzyme catalyzes a net γ-elimination reaction from OSHS to yield succinate, α-ketobutyrate, and ammonia (eq 2). The turnover rate for the γ-elimination reaction is only 5-10-fold slower than the γ-replacement reaction (Kaplan & Flavin, 1966; Johnston et al., 1979a; Holbrook et al., 1990). The enzyme also supports certain β-elimination, β-replacement,

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[1] Abbreviations: PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; OSHS, *O*-succinyl-L-homoserine; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; RSSF, rapid-scanning stopped-flow; SWSF, single-wavelength stopped-flow; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetate; DSS, 2,2-dimethyl-2-silapentene-5-sulfonate-2,2,3,3-d_4; L-Cys, L-cysteine; DTE, dithioerythritol.

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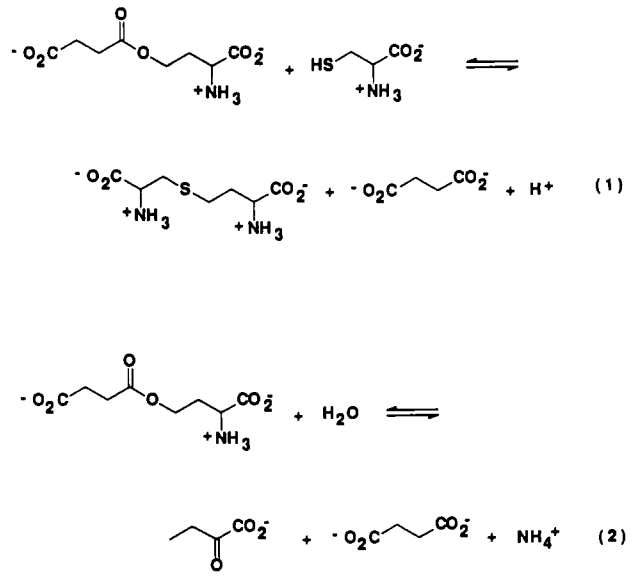


and proton-exchange reactions at both the α- and β-carbons of suitable amino acid substrate analogues (Guggenheim & Flavin, 1969a,b).

The microbial enzymes from *Salmonella typhimurium* and *E. coli* have been the most extensively studied and characterized (Johnston et al., 1979a,b; Holbrook et al., 1990). Both appear to be very closely related (Holbrook et al., 1990). Each is a tetramer of identically sized subunits (M_r = 40 000) with four PLP cofactors per tetramer. The reported spectral and kinetic behavior of these enzymes is nearly identical.

Construction of a strain of *E. coli* that overproduces cystathionine γ-synthase has allowed purification of large quantities of the enzyme (Holbrook, 1984). This has made possible the detailed UV–visible spectral and kinetic characterization of the enzyme by rapid-scanning stopped-flow (RSSF) and single-wavelength stopped-flow (SWSF) spectroscopy. Using these techniques, we have exploited the chromophoric properties of the pyridoxal phosphate cofactor to probe the chemical transformations that occur during catalysis. As will be shown herein, observation of the time-resolved, pre-steady-state spectral changes in the region between 280 and 550 nm, where most PLP species absorb, allows direct detection of important catalytic intermediates.

These experiments reveal the presence of a previously undetected reaction intermediate that absorbs in the 300-nm region of the spectrum. This intermediate, which is likely a pyridoxamine derivative of the cofactor, is common to both the γ-replacement and γ-elimination pathways. These observations have important mechanistic implications for the reactions catalyzed by cystathionine γ-synthase and may have a broader general significance for those PLP-requiring enzymes that, of necessity, generate carbanions at the β-position of amino acid substrates.

Experimental Procedures

Materials. *O*-Succinyl-L-homoserine, L-cysteine hydrochloride, L-allylglycine, NADH, DSS, DTE, and LDH from rabbit muscle were purchased from Sigma. D_2O was purchased from ICN Biochemicals.

Enzyme and Enzyme Assays. Cystathionine γ-synthase was isolated from *E. coli* strain pLLH346/RG326 as described by Holbrook et al. (1990). The enzyme activity was determined via an assay that couples the cystathionine γ-synthase catalyzed production of α-ketobutyrate from OSHS to the LDH-catalyzed reduction of α-ketobutyrate by NADH. Protein concentration was determined by measuring the absorbance at 422 nm (ϵ_{422} = 33 850 M^{-1} cm^{-1} for the enzyme tetramer; Holbrook et al., 1990).

Equilibrium Spectral Measurements of Cystathionine γ-Synthase with L-Alanine and L-Allylglycine. Static UV–visible spectral measurements were obtained with a Hewlett-Packard 8450A spectrophotometer. Equilibrium spectra of the cystathionine γ-synthase–L-allylglycine complex were obtained by adding sequential aliquots of a 300 mM L-allylglycine and 6.25 μM cystathionine γ-synthase solution to a second 6.25 μM enzyme solution and allowing the sample to equilibrate. This approach is viable since L-allylglycine is not readily degraded to an α-keto acid by the enzyme (see Results).

Synthesis and Isolation of γ-Methylcystathionine. A 5-mL solution of 15 mM L-allylglycine, 10 mM L-cysteine, 10 mM DTE, and 0.7 μM cystathionine γ-synthase was allowed to react for 4 h in 0.1 M KPO_4 and 1 mM EDTA, pH 7.2 at 25 °C. The reaction was terminated by adjusting the pH of the solution to 4.0 with glacial acetic acid. The sample was applied to a 1.0 × 1.6 cm Bio-Rad AG50 W8 column and eluted with a 0.01 M Na_2PO_4 gradient from pH 4.0 to 9.0. The 2-mL fractions were assayed by ninhydrin spot tests. Fractions that contained amino acids were further analyzed by thin-layer chromatography on Whatman silica gel plates. (R_f for γ-methylcystathionine is 0.11 in 1-butanol/acetic acid/H_2O 12:3:5). Fractions containing γ-methylcystathionine were pooled and lyophilized. The amino acid was reconstituted in 1 mL of D_2O for ^1H NMR analysis.

^1H NMR Analysis. All ^1H NMR spectra were recorded on a Nicolet 300-MHz spectrophotometer. The reaction mixture for the exchange reaction between substrate hydrogens and solvent deuterium contained 10 mM L-allylglycine, 1 μM enzyme, and DSS in D_2O as the solvent. The final volume of the reaction mixture was 2.1 mL. Solutions were prepared by dissolving the compounds and enzyme in D_2O, lyophilizing, and redissolving in D_2O.

Single-Wavelength Stopped-Flow Spectrophotometry. Single-wavelength pre-steady-state kinetic measurements were performed with a Durrum Model D-110 rapid-mixing stopped-flow spectrophotometer interfaced for on-line computer data acquisition and analysis (Drewe & Dunn, 1985). All concentrations refer to conditions after mixing.

Rapid-Scanning Stopped-Flow (RSSF) Spectrophotometry. The RSSF spectrophotometer used in these studies employed elements of the Durrum D-110 rapid-mixing stopped-flow spectrometer and a Princeton Applied Research (PAR) OMA-III multichannel analyzer with a 1463 detector-controller card and a 1214 photodiode array detector. The hardware for this system has been previously described (Koerber, 1983).

For a typical experiment, a 100% transmission reference spectrum (defined as the light transmitted through the buffer solution used) and the diode array dark-current spectrum are first collected and stored. By use of these spectra, the rapid-scanning data collected are converted to absorbance and stored on floppy disk. The experiments reported herein used 512 pixels for a repetitive scan time of 8.9 ms and a wavelength resolution of ±1 nm. All concentrations refer to conditions after mixing.

Results

γ-Replacement and γ-Elimination Reactions Catalyzed by Cystathionine γ-Synthase from O-Succinyl-L-homoserine. The time-dependent spectra and single-wavelength time courses for the pre-steady-state phase of the OSHS γ-replacement and γ-elimination reactions are shown in Figures 1 and 2. In the absence of substrate, the native enzyme

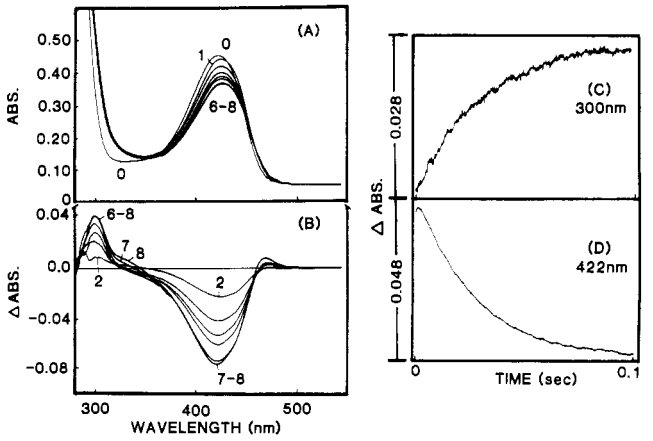


FIGURE 1: Rapid-scanning stopped-flow spectra and difference spectra of the γ-replacement reaction with OSHS and L-cysteine. Before mixing, the enzyme (syringe 1) and both substrates (syringe 2) were incubated separately. All concentrations refer to those immediately after mixing: [OSHS] = 10 mM, [L-cysteine] = 10 mM, [DTE] = 5 mM (DTE is not a substrate for the γ-replacement reaction; Kaplan & Flavin, 1966), [cystathionine γ-synthase] = 6.25 μM, 0.1 M potassium phosphate, 1 mM EDTA, pH 7.2 at 25 °C. (A) RSSF data were obtained by collection of 79 sequential scans with a repetitive scan time of 8.9 ms. The scans shown are a representative subset of the 79 collected for each reaction. The trace designated 0 is a spectrum of the enzyme in the absence of substrates. The initiation of scanning occurred at 1.3 ms after flow stopped. The scan acquisition times were 1.3, 10.2, 19.1, 28., 36.9, 72.5, 250.5, and 695.5 ms after flow stopped. (B) Difference spectra were computed as $(\text{scan})_i-(\text{scan})_1$ from the data presented in (A) (see footnote 2). (C, D) Spectra collected in single-wavelength stopped-flow experiments under conditions identical with the RSSF experiments.

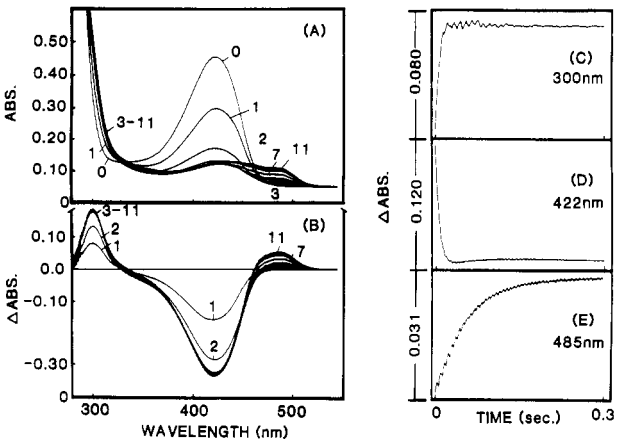


FIGURE 2: Rapid-scanning stopped-flow spectra, difference spectra, and single-wavelength stopped-flow time courses for the γ-elimination reaction of cystathionine γ-synthase with OSHS. Data were obtained as described in Figure 1. All concentrations refer to conditions immediately after mixing: [OSHS] = 10 mM, [cystathionine γ-synthase] = 6.25 μM, 0.1 M potassium phosphate, 1 mM EDTA, pH 7.2 at 25 °C. (A) RSSF spectra. Trace 0 is the spectrum of the enzyme in the absence of substrates. The initiation of scanning occurred 1.3 ms after flow stopped. Scans were collected at 1.3, 10.2, 19.1, 28.0, 36.9, 72.5, 117.0, 161.5, 250.5, 392.9, and 695.5 ms after flow stopped. (B) Difference spectra were computed as $(\text{scan})_i-(\text{scan})_0$ from the data presented in (A). Single-wavelength time courses (C–E) were collected in single-wavelength stopped-flow experiments under conditions identical with those described for (A).

displays an absorption band with a maximum at 422 nm. This band is generally characteristic of the pyridoxal phosphate cofactor covalently bound to the apoenzyme via a Schiff base linkage to the ε-amino group of a lysine side chain. Under replacement reaction conditions, the spectral changes that occur upon rapid mixing of equal concentrations of OSHS and L-Cys (10 mM) (Figure 1A) give evidence for the occurrence of three relaxations ($1/\tau_1^R \gg 1/\tau_2^R \gg 1/\tau_3^R$) in the pre-steady-state phase of the reaction. During the mixing dead time, there is a 3-nm shift in the absorbance maximum to 425 nm. This relaxation ($1/\tau_1^R$) is followed by a second relaxation ($1/\tau_2^R$) characterized by a decrease in absorbance and further red shift to 427 nm. Concomitant with this process is the accumulation of a previously undetected intermediate absorbing in the 300-nm region of the spectrum. The large initial increase at 300 nm between the enzyme spectrum (spectrum 0) and the first spectrum collected after mixing (spectrum 1) is due to the introduction of L-Cys into the reaction mixture.[2] The accumulation of a new intermediate in the 300-nm region $1/\tau_2^R$ is clearly shown in the difference spectra of Figure 1B. These spectra were obtained by subtracting the first spectrum obtained after mixing (spectrum 1, Figure 1A) from all subsequent spectra. The apparent absorption maximum obtained from these difference spectra is 300 nm. (Subsequent reference to this intermediate will refer to this apparent absorption maximum.) Noticeable in the difference spectra is a small but significant increase in absorbance at 340 nm which occurs in the third relaxation ($1/\tau_3^R$) after accumulation of the 300-nm species is nearly complete (spectra 7 and 8, Figure 1A). Cystathionine does not absorb above 250 nm, and product formation during the pre-steady-state phase of the reaction is insignificant; therefore, product accumulation does not obscure absorbance changes at 340 nm.

The single-wavelength time courses at 300 and 422 nm are shown in parts C and D of Figure 1. The time course at 422 nm is biphasic. There is a rapid initial increase in absorbance ($1/\tau_1^R > 500\ s^{-1}$) which is consistent with the time-unresolved red shift observed in the RSSF spectra (Figure 1A). This rapid relaxation is followed by a monophasic decrease ($1/\tau_2^R = 50\ s^{-1}$) that is concomitant with the formation of a new 300-nm-absorbing species. Both time courses yield the same observed rate constant for $1/\tau_2^R$ and, therefore, are tightly coupled processes. A third relaxation is detected as an absorbance increase at 340 nm ($1/\tau_3^R = 5\ s^{-1}$) and is ~10-fold slower (data not shown). As the reaction nears completion, the original enzyme spectrum is recovered and all time courses revert toward initial absorbance values (data not shown). This indicates all species described are transients.

Noticeably absent from these spectra is the accumulation of any long-wavelength-absorbing reaction intermediates.[3] This behavior is different from that of steady-state spectra previously reported for the γ-replacement reaction with the *Salmonella* enzyme (Johnston et al., 1979a). In previous experiments, after initiation of the γ-elimination reaction with

[2] The thiolate anion of L-Cys displays an absorption maximum at 250 nm due to an n,n^* electronic transition. The extinction of this band is large enough that for a 10 mM cysteine solution, pH 7.2, there is a significant absorbance in the 300-nm region of the spectrum. For this reason, there is a large initial absorbance increase between the native enzyme spectrum and the first spectrum collected after mixing during the γ-replacement study shown in Figure 1A.

[3] The difference spectra shown in Figure 1B reveal an increase in absorbance at 470 nm, particularly in spectra 7 and 8. This increase is very small, but reproducible. It may be caused by the red shift in the absorbance maximum observed from the formation of the external aldimine between the substrate or product and the cofactor (compare spectra 0 and 1, Figure 1A). Alternatively, it may be caused by the accumulation of a quinonoidal species predicted to exist along the reaction pathway (Scheme II, structures II and VII). Quinonoidal species generally have extinction coefficients between 30 000 and 50 000 $M^{-1}\ cm^{-1}$. Therefore, this would represent a very small amount of this species present. Experiments are in progress to further investigate this observation.

OSHS, a limiting concentration of L-Cys was introduced into the reaction mixture. These conditions result in the initial accumulation of a 485-nm band (see the data presented for the γ-elimination reaction, Figure 2A) which persists after the addition of L-Cys. We have been able to reproduce this behavior with the *E. coli* enzyme. Steady-state spectra at 25 °C indicate that the order of substrate addition is important in determining whether or not a 485-nm chromophore is present. As shown in Figure 1A, simultaneous addition of substrates produces no 485-nm species. Even upon simultaneous mixing with limiting L-Cys, no 485-nm band appears until L-Cys is exhausted from the reaction mixture (data not shown).

The pre-steady-state absorbance changes that accompany the γ-elimination reaction from OSHS (Figure 2A) are markedly different from those of the γ-replacement reaction (Figure 1A). Three relaxations with rates $1/\tau_1^E > 1/\tau_2^E > 1/\tau_3^E$ are evident in Figure 2. Rapid mixing with only OSHS, at the same concentration used for the γ-replacement reaction, results in a rapid and nearly quantitative bleaching of the 422-nm peak in $1/\tau_1^E$ (viz. Figures 2A and 1D). This loss of absorbance is accompanied by a concomitant accumulation of the 300-nm intermediate (Figure 2A–C). The fast relaxations at both 300 and 422 nm have the same observed rate constant of 150 s^{-1} (which is 3-fold faster than for the γ-replacement reaction) and exhibit the same concentration dependence of rates on OSHS (data not shown). The tight coupling between the two processes at 422 and 300 nm (compare parts C and D of Figure 2) is clearly seen in the difference spectra shown in Figure 2B. Under these conditions the amplitude changes are much larger than those observed during the γ-replacement reaction (compare Figures 1B and 2B). On a longer time scale there is a biphasic increase in absorbance at 485 nm ($1/\tau_2^E \sim 18$ s^{-1}, $1/\tau_3^E \sim 0.5$ s^{-1}) (Figure 2A,E). This change is actually due to the slow appearance of a broad absorbance increase between 440 and 500 nm consisting of at least two overlapping bands with apparent λ_{max} of 460 and 485 nm.[4] There is very little absorbance increase in this region of the spectrum until the fast phase of the reaction is nearly complete (compare parts D and E of Figure 2). The third relaxation, $1/\tau_3^E$, is evident as the slowest process detected in Figures 2C–E.

Reaction of Cystathionine γ-Synthase with L-*Alanine and* L-*Allylglycine.* Cystathionine γ-synthase catalyzes α- and β-proton exchange for deuteron in D_2O in a number of amino acid substrate analogues (Guggenheim & Flavin, 1969b). To this list may be added the γ-δ olefinic amino acid L-allylglycine. Figure 3B presents ^1H NMR spectra collected at various times after the addition of L-allylglycine to catalytic amounts of the enzyme in D_2O. After 240 min, the α-proton signal has nearly disappeared. The original multiplet arising from the two nonequivalent β-protons collapses into a doublet, and the peak intensity decreases. Kinetic analyses reveal that the α-proton and one β-proton undergo exchange with solvent at roughly the same rate (data not shown). Cystathionine γ-synthase also catalyzes solvent exchange of the second β-proton, but at a much slower rate. This behavior is similar to that previously reported for L-homoserine (Posner & Flavin, 1972a). Signals arising from the olefinic protons of the substrate undergo no appreciable changes (data not shown).

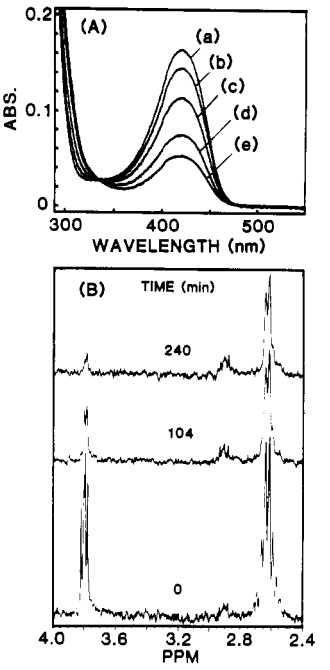


FIGURE 3: Equilibrium titration of 6.25 μM cystathionine γ-synthase with L-allylglycine. Spectra A–E were measured for solutions containing 0, 1.98, 5.83, 18.2, and 465.2 mM L-allylglycine, respectively, in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.2 at 25 °C. (B) 300-MHz ^1H NMR collected during the reaction of 1 μM cystathionine γ-synthase with 10 mM L-allylglycine in D_2O at 0, 104, and 240 min after the addition of enzyme.

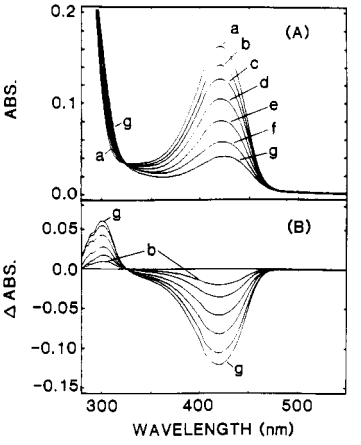


FIGURE 4: (A) Equilibrium titration of 5 μM cystathionine γ-synthase with L-alanine. Spectra a–g were measured for solutions containing 0, 0.038, 0.075, 0.15, 0.3, 0.6, and 1.2 M L-alanine, respectively, in 0.1 M potassium phosphate and 1 mM EDTA, pH at 25 °C. The final volume for each measurement was 1 mL. (B) Difference spectra were calculated by subtraction of the spectrum of 5 μM cystathionine γ-synthase only (part A, spectrum a) from all subsequent spectra collected in the presence of L-alanine (part A, spectra b–g).

The UV–visible spectral changes that occur during the proton-exchange reaction are shown in Figure 3A. Titration of the enzyme with allylglycine results in a large decrease in absorbance at 422 nm but no apparent shift in peak position. There is a corresponding accumulation of a new chromophore at 300 nm. These spectra exhibit an apparent isoabsorptive point at 335 nm. *No long-wavelength-absorbing intermediates accumulate during the proton-exchange reaction with* L-*allylglycine.* These spectral changes are strikingly similar to those observed during $1/\tau_2^E$ of the γ-elimination reaction from OSHS (Figure 2A).

[4] The overlapping bands at 460 and 485 nm are also observed for a number of substrate analogues (Johnston et al., 1979a). Time courses at 460 and 485 nm give identical observed rates for the appearance of these bands. Though these bands could be derived from two chemically distinct intermediates, at present, we favor the explanation that these bands arise from rapid interconversion between different tautomeric forms or protonation states of a single species.

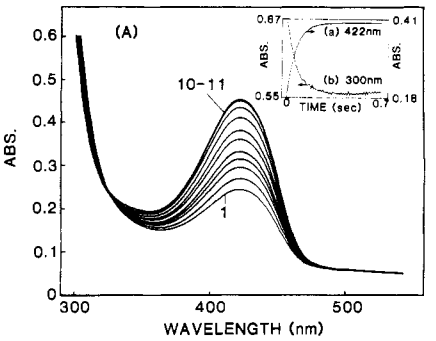


FIGURE 5: Rapid-scanning stopped-flow spectra for the reaction of cystathionine γ-synthase with L-allylglycine and L-cysteine. Before mixing, the enzyme was placed in one stopped-flow syringe, while L-cysteine was placed in the other. Both syringes contained equal concentrations of L-allylglycine. The concentrations immediately after mixing were [cystathionine γ-synthase] = 7.08 μM, [L-allylglycine] = 28 mM, [L-cysteine] = 10 mM, and [DTE] = 5 mM in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.2 at 25 °C. Initiation of scanning occurred 2.3 ms after flow had stopped. Data shown are representative scans from a 79 scan data set collected at a repetitive scan time of 8.9 ms. The scan acquisition times are 2.3, 11.2, 20.1, 29.0, 37.9, 55.7, 73.5, 109.1, 162.5, 289.6, and 696.5 ms after flow stopped. The inset to (A) presents single-wavelength time courses taken from the entire set of scans at (a) 300 and (b) 420 nm. The left and right oridinates correspond to the absorbance values at 300 and 420 nm, respectively.

Very similar changes occur in the reactions of the substrate analogues L-alanine (Figure 4) (Guggenheim & Flavin, 1971; Holbrook, 1984) and *trans*-4-methoxy-L-vinylglycine (data not shown). As shown in Figure 4A, L-alanine produces quenching of the 422-nm band with a compensatory increase in absorbance at 300 nm. There is an apparent isoabsorptive point at 325 nm. These changes are clearly seen in the difference spectra (Figure 4B). L-Alanine also undergoes proton exchange at both α- and β-positions in D_2O (Guggenheim & Flavin, 1971). *trans*-4-Methoxy-L-vinylglycine also results in the formation of a 300-nm species. The spectral changes are identical with those reported for the reaction with methionine γ-lyase (Johnston et al., 1981).

The ability of cystathionine γ-synthase to catalyze α- and β-proton exchange in L-allylglycine (Figure 3B) indicates the binary complex between L-allylglycine and the enzyme is formed reversibly. In marked contrast to OSHS, however, L-allylglycine does not react readily to give α-keto acid products. After several hours of incubation of the enzyme with allylglycine, no α-keto acids could be detected either by accumulation of a characteristic UV absorbance band, by use of a coupled assay with LDH, or in the NMR spectra. Nonetheless, this adduct is reactive toward L-Cys. Figure 5 shows pre-steady-state RSSF spectral changes for the reaction of L-Cys with cystathionine γ-synthase preequilibrated with L-allylglycine. There is a rapid loss of the 300-nm chromophore and a concomitant increase at 422 nm with an apparent isoabsorptive point at 335 nm during the single relaxation detected ($1/\tau^{AG}$). As with the γ-elimination and γ-replacement reactions from OSHS, the changes that occur at 300 and 422 nm with the L-allylglycine system appear to be tightly coupled (Figure 5, inset). The changes at both wavelengths are monophasic and display the same observed rate constants ($1/\tau^{AG} = 30\ s^{-1}$). Analogous to the third relaxation of the γ-replacement reaction between OSHS and L-Cys, there does appear to be a slow accumulation of an intermediate absorbing at 340 nm in this reaction (Figure 5, spectrum 11). Again, no long-wavelength-absorbing intermediates accumulate during the course of this reaction.

Isolation of the reaction product (see Experimental Procedures) reveals the formation of a new amino acid. Structural determination by ^1H NMR establishes that the reaction product is the cystathionine analogue γ-methylcystathionine.

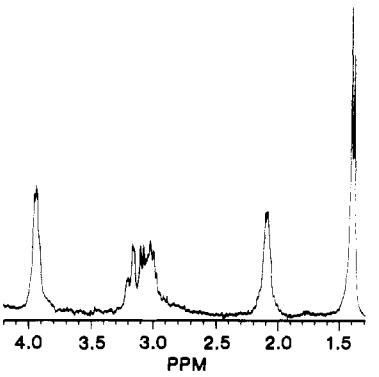


FIGURE 6: 300-MHz ^1H NMR spectrum of γ-methylcystathionine in D_2O. The product was isolated (see Experimental Procedures) from a reaction mixture containing 15 mM L-allylglycine, 10 mM L-cysteine, 10 mM DTE, and 0.7 μM cystathionine γ-synthase in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.2 at 25 °C.

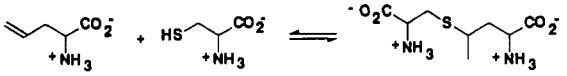

(3)

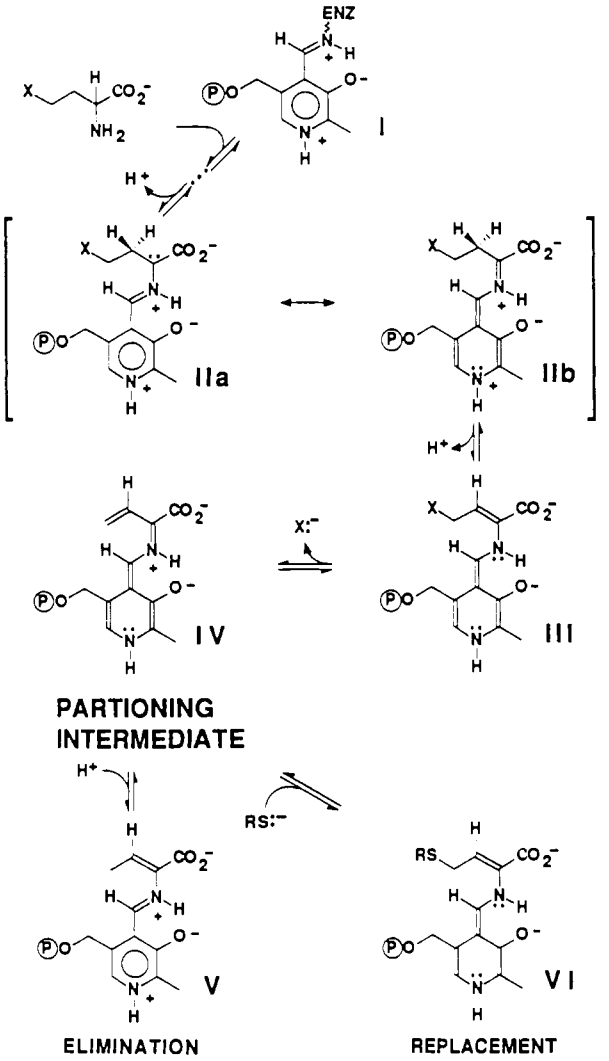
Figure 6 shows four separate envelopes of resonances centered respectively at 3.95, 3.1, 2.1, and 1.35 ppm; integration gives the relative ratios for the areas under each envelope as 2:3:2:3, respectively. The quartet at 3.95 ppm arises from the two α-protons. The complex multiplet at 3.1 ppm consists of overlapping resonances from the single γ-proton and the two β-protons directly adjacent to the sulfur atom. The remaining two β-protons appear as a complex multiplet at 2.1 ppm. A key structural feature of the spectrum is the appearance of a methyl doublet at 1.35 ppm. Therefore, analogous to OSHS, the enzyme renders L-allylglycine susceptible to Michael addition at the γ-carbon of the substrate.

Discussion

Recent proposals for the reaction mechanisms of PLP-dependent γ-elimination and γ-replacement reactions have focused on the possible involvement of a β-γ unsaturated α-imino *p*-quinonoid species (vinylglycine quinonoid, IV, Scheme I) as an important reaction intermediate. For cystathionine γ-synthase, which catalyzes both types of reactions from the physiological substrate OSHS, it has been proposed that there must exist a number of common steps culminating in the elimination of the γ-substituent and the formation of the vinylglycine quinonoid as the key partitioning intermediate between the replacement and elimination pathways (Johnston et al., 1979a). The details of this mechanistic proposal are outlined in Scheme I (Davis & Metzler, 1972; Walsh, 1979). Support for this stepwise reaction pathway is based primarily on the ability of cystathionine γ-synthase to catalyze proton exchange at both the α- and β-positions for a variety of amino acid substrates (Guggenheim & Flavin, 1969b) and the ability of vinylglycine to enter the reaction sequence and undergo either nucleophilic addition at the γ-carbon to yield L,L-cystathionine on protonation at the γ-carbon resulting in the formation of α-ketobutyrate and ammonia.

The mechanism outlined in Scheme I contains two inherent contradictions. (1) For catalysis to occur via this mechanism, the enzyme must generate a resonance delocalized β-carbanion (III) (by removal of a C-β-proton) which subsequently assists in the elimination of the γ-substituent. Therefore, the enzyme must activate two carbon-bound protons for removal, the

Scheme I: Proposed Mechanism for the γ-Replacement and γ-Elimination Reactions Catalyzed by Cystathionine γ-Synthase [Adapted from Walsh (1979)][a]



[a] The vinylglycine quinonoidal species (IV) is hypothesized to be the partitioning intermediate between the pathways. Structures Ia and IIb, reasonance structures of the quinonoid formed by removal of the α-carbon, emphasize that the α-carbon must retain carbanionic character.

C-α-proton followed by the C-β-proton. These protons generally have pK_as in solution approaching 30–50. The α-proton is labilized by formation of an external aldiminium ion which stabilizes the subsequent α-carbanion by extensive electron delocalization with the pyridine ring of the cofactor (IIa and IIb). The α-carbon must retain a significant amount of electron density for protonation to be reversible. According to Scheme I, a β-proton must be removed from a species that already has an α-carbon with carbanionic character. This situation would likely serve to increase the pK_a of the β-proton and hence would act contrary to efficient catalysis. (2) The vinylglycine quinonoid (IV), a single structural intermediate, is required to display both electrophilic and nucleophilic behavior at the γ-carbon. The functional duality of this intermediate is mediated solely by the presence or absence of an appropriate thiol nucleophile. For these reasons we view this mechanistic proposal to be chemically implausible.

The spectral studies reported herein do not support the mechanism outlined in Scheme I. As will be shown below, the detection of an intermediate(s) absorbing in the 300-nm region of the spectrum during both the γ-replacement and γ-elimination reactions provides the first direct evidence for the existence of a kinetically competent pyridoxamine derivative of the cofactor during cystathionine γ-synthase catalysis.

*γ-Replacement Reaction from O-Succinyl-*L*-homoserine and* L*-Cysteine.* Scheme I predicts the existence of a number of reaction intermediates with extended conjugation (II, IV, and V). These species are expected to absorb at wavelengths substantially greater than the 422-nm band of the internal aldimine (I). The α-quinonoidal species (II) have been observed in a number of PLP-dependent enzymatic reactions (Davis & Metzler, 1972). The characteristic absorption bands for these species are generally observed between 460 and 505 nm (Davis & Metzler, 1972; Kallen et al., 1985). Model studies of the β-γ unsaturated vinylglycine quinonoid (IV) in solution indicate this species has an absorption maximum at 550 nm (Karube & Matsushima, 1977; Johnston et al., 1981). RSSF spectra collected during the pre-steady-state phase of the γ-replacement reaction between OSHS and L-cysteine (Figure 1A) reveal that no such long-wavelength-absorbing species accumulate (see footnote 3). The rapid shift of the external aldimine spectrum from 422 nm to a species with λ_{max} = 425 nm (compare spectra 0 and 1, Figure 1A) is similar to that observed during the pre-steady-state reaction for tryptophan synthase with L-serine (Drewe & Dunn, 1985). This shift is most reasonably attributed to the rapid formation of a Schiff base (external aldimine) between the PLP cofactor and the amino group of the substrate. This assignment is consistent with the very rapid initial increase in absorbance at 422 nm ($1/\tau_1^R > 500\ s^{-1}$) observed in SWSF time courses (Figure 1D). The subsequent disappearance of the 425-nm species (Figure 1A, spectra 2–8) is tightly coupled to the appearance of a new species absorbing in the 300-nm region of the spectrum. The accumulation of this chromophore is clearly seen in the difference spectra of Figure 1B.

*γ-Elimination Reaction from O-Succinyl-*L*-homoserine.* Elimination of the γ-substituent from OSHS is an obligatory event for both the γ-replacement and γ-elimination pathways. Therefore, it is reasonable to predict that both pathways share a number of common intermediates before diverging. However, the pre-steady-state spectral changes that accompany the γ-elimination reaction from OSHS are markedly different from the spectral changes of the γ-replacement reaction (compare Figures 1A and 2A). The amplitude changes for the rapid disappearance of the internal aldimine (λ_{max} = 422 nm) and the concomitant appearance of the 300-nm species are much larger, and a new intermediate absorbing at 485 nm (not detected in the replacement reaction) accumulates on a much slower time scale (Figure 2E). This 485-nm species previously has been described in steady-state studies by Guggenheim and Flavin (1971) and Johnston et al. (1979a). Johnston et al. (1979a) have suggested an α-aminocrotonate structure for this species (V, Scheme I). We agree with this assignment for the following reasons: (1) The 485-nm absorbance band is consistent with the extent of conjugation for this intermediate. (2) The 485-nm intermediate may be formed directly by cystathionine γ-synthase catalyzed β-elimination from *O*-succinyl-L-serine (Guggenheim & Flavin, 1971), β-chloroalanine, and β-halo-α-aminobutyrates (Johnston et al., 1979a; data not shown). Therefore, the extent of the conjugated π-system cannot involve the γ-carbon of the substrate. This assignment is further supported by the finding that the wavelength maximum of the 485-nm species is located at a much shorter wavelength than is the long-wavelength band of the highly conjugated vinylglycine quinonoid reported from model studies (Karube & Matsushima, 1977; Johnston et al.,

1981). (3) The 485-nm intermediate appears to be committed along the γ-elimination pathway. It accumulates on a much slower time scale than does the 300-nm species and is observed only during the γ-elimination reaction. The 485-nm species formed directly by β-elimination from β-halo-α-aminobutyrate substrates readily react to yield α-ketobutyrate, but these compounds do not support the synthesis of cystathionine in the presence of cysteine (Johnston et al., 1979a).

Reaction of Cystathionine γ-Synthase with L-*Alanine and* L-*Allylglycine.* One of the inherent difficulties in studying the cystathionine γ-synthase reaction mechanism has been the inability to separately investigate each of the individual half-reactions starting from OSHS. This limitation is due to the rapid conversion of OSHS to succinate, α-ketobutyrate, and ammonia (eq 2) in the absence of L-Cys. The use of the substrate analogues L-alanine and L-allylglycine provides a viable approach to circumvent this problem by separating the reaction into two half-reactions that may be studied without complicating side reactions.

Addition of either L-alanine or L-allylglycine to the reaction mixture produces spectral changes similar to the spectral changes that occur during the fast phase of the γ-elimination reaction from OSHS (compare Figures 3A and 4 with 2A); i.e., there is a large loss in absorbance at 422 nm concomitant with the accumulation of a chromophore absorbing at 300 nm. As observed for the γ-replacement reaction from OSHS, neither the rapid kinetic spectra (data not shown) nor the spectra at equilibrium (Figures 3 and 4) for the L-allylglycine or L-alanine system show the accumulation of any long-wavelength-absorbing intermediates. This is in contrast to the γ-elimination reaction from OSHS (compare Figures 2A and 3A).

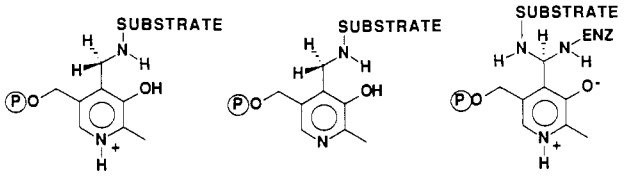
Of particular mechanistic importance is the observation that, despite the absence of any long-wavelength-absorbing species (Figures 3A and 4), in D_2O, L-allylglycine and L-alanine undergo an enzyme-catalyzed exchange of protons at both the α- and β-carbons (Figure 3B) (Guggenheim & Flavin, 1971). The predominant enzyme species present under these conditions is one absorbing at 300 nm.

Reactivity of the 300-nm-Absorbing Intermediate. The one distinctive feature common to the spectral changes for all reactions described is the appearance of a kinetically competent intermediate absorbing in the 300-nm region of the spectrum. This 300-nm species appears to be very reactive toward L-cysteine in the γ-replacement reaction from OSHS. Double-push stopped-flow experiments have been performed in which the enzyme is initially mixed with OSHS, aged for 55 ms, and then mixed with L-Cys (Holbrook, 1984). The aging time is long enough for nearly complete formation of the 300-nm intermediate with bleaching of the 422-nm absorbance (Figure 2A, spectra 0–5). Very little of the 485-nm intermediate forms within this time frame. Subsequent mixing with L-Cys results in a very rapid recovery of 422-nm absorbance within the mixing dead time of the instrument, indicating that it is indeed the 300-nm species that is reacting directly with L-Cys. Furthermore, the 300-nm species produced in the L-allylglycine reaction is reactive toward the thiol functional group of L-Cys (Figure 5). The spectral changes that take place upon addition of L-Cys show a rapid loss of absorbance at 300 nm with a concomitant increase at 422 nm ($1/\tau^{AG}$ = 30 s^{-1}). The product produced, γ-methylcystathionine (Figure 6), establishes that the enzyme activates allylglycine for nucleophilic attack at the γ-carbon in a mechanism undoubtedly analogous to the mechanism of the γ-replacement reaction with OSHS as a substrate.

Assignment of Pyridoxamine Species as Obligatory Intermediates in the γ-Replacement and γ-Elimination Reactions. Model studies in aqueous solution reveal that neutral or cationic PLP derivatives that have tetrahedral geometry (sp^3 hybridization) at the C-4′ carbon of the cofactor will absorb in the 300-nm region of the spectrum (Metzler et al., 1973; Kallen et al., 1985). Structures XVII–XIX are intermediates consistent with this behavior. Structure XIX is a geminal diamine intermediate formed during transaldimination between the substrate and cofactor. Structures XVII and XVIII are formed via a net 1,3 prototropic shift between the α-carbon of the substrate and the C-4′ carbon of the cofactor.

It is unlikely that the 300-nm species is an unusually stable form of the geminal diamine intermediate (XIX). The time-unresolved 425-nm red shift occurring during the γ-replacement reaction (Figure 1A) is attributed to the rapid formation of an external aldimine (VIII, Scheme II). This is consistent with the SWSF time courses at 422 nm (Figure 1D). Formation of the geminal diamine species (XIX) must precede this step, whereas accumulation of the 300-nm-absorbing species clearly occurs afterward. Furthermore, it is the 300-nm species that is highly reactive toward nucleophilic attack by L-Cys, whereas the geminal diamine would not be reactive.[5]

The observed kinetic behavior, UV–visible properties, and mechanistic imperatives of the reaction are consistent with the intermediacy of one or more pyridoxamine derivatives of the cofactor (XVII and XVIII) for the reactions catalyzed by



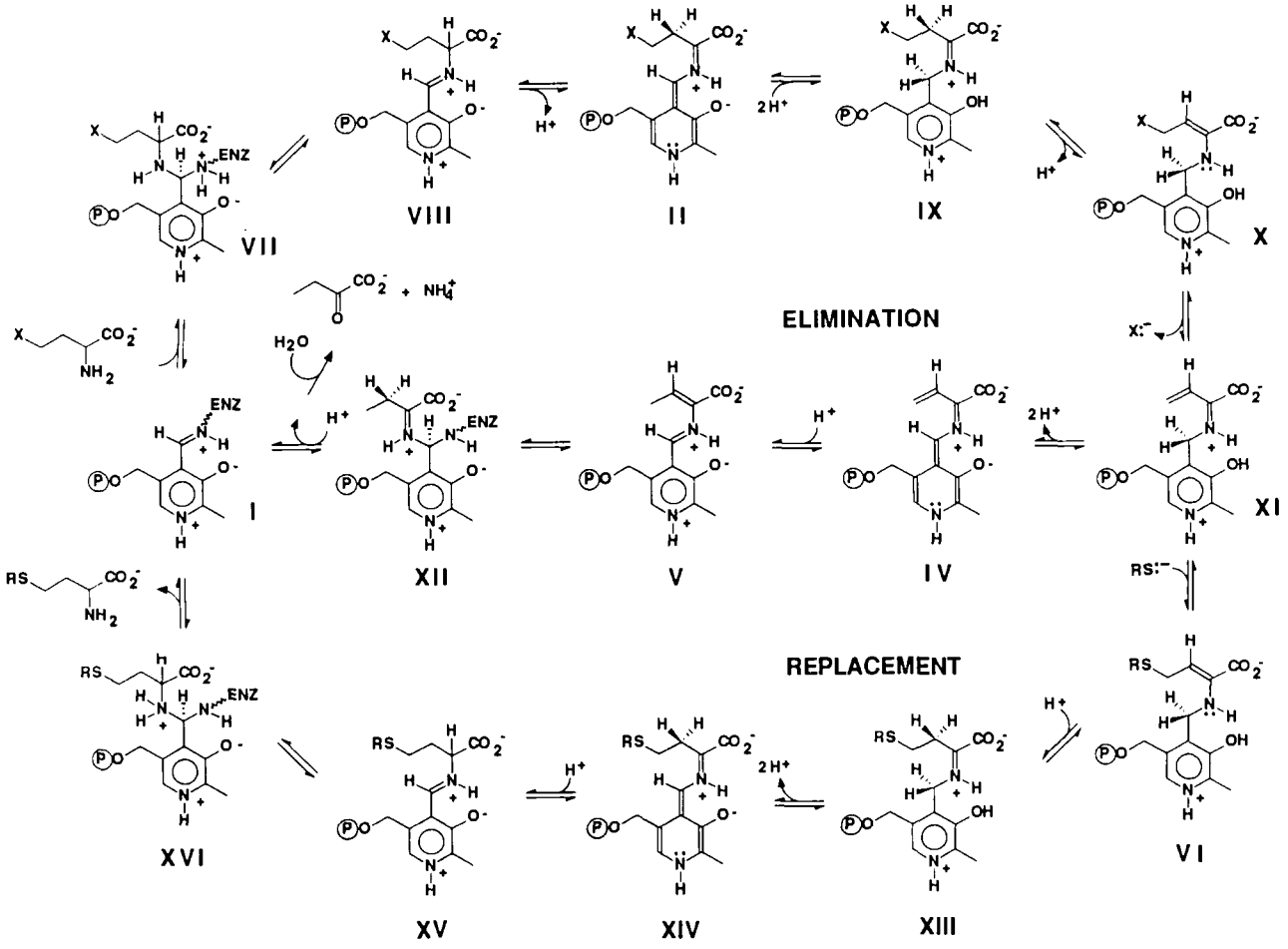
XVII XVIII XIX

cystathionine γ-synthase. It is such a derivative that is the final common intermediate between the γ-replacement reaction and the γ-elimination reaction. Other workers (Guggenheim & Flavin, 1971; Johnston et al., 1979a) have speculated on the existence of pyridoxamine intermediates, and data presented in their papers are not inconsistent with such species. Here we provide the first direct evidence for these intermediates in the reaction mechanism of cystathionine γ-synthase.

Generalized Reaction Mechanism. A mechanism utilizing a PMP form of the cofactor is outlined in Scheme II [adapted from Jencks (1969); Posner & Flavin, 1972b]. Similar to the

[5] We speculate that the geminal diamine intermediate absorbs in the 340-nm region for the following reasons: (1) During the γ-replacement reaction, there is a transient accumulation of an intermediate absorbing at 340 nm (Figure 1A, spectra 7 and 8) which decays as the reaction nears completion (data not shown). The rate of formation ($1/\tau_3^R \simeq 5$ s^{-1}) of this species is similar to that of the turnover number of the enzyme at pH 7.2 (T.O.N. = 10 s^{-1}; Holbrook, 1984). This species also appears to be present in the γ-replacement reaction between L-Cys and L-allylglycine (Figure 5B, spectrum 11). (2) Reduction of native enzyme with $NaBH_4$ results in the formation of a C-4′ tetrahedral species of the cofactor covalently linked to the enzyme via the ε-amino group of a lysine residue. This species absorbs at 340 nm and is highly fluorescent (λ_{em} = 390 nm) (Holbrook, 1984). (3) The reaction of L-alanine with the enzyme gives at least two species, one characterized by a 300-nm spectral band that is not fluorescent and the other (apparently present in small amounts) detected by fluorescence ($\lambda_{max,ex}$ = 340 nm, $\lambda_{max,em}$ = 390 nm). This species displays fluorescent behavior that is very similar to that of the $NaBH_4$-reduced holoenzyme (data not shown). Therefore, we conclude that this 340-nm species is the geminal diamine that occurs during transaldimination between the substrate or product and the PLP cofactor (IX).

Scheme II: Generalized Reaction Mechanism for PLP-Dependent γ-Elimination and γ-Replacement Reactions Involving Pyridoxamine Derivatives of the Cofactor[a]



[a] The pyridoxamine form of the β-γ unsaturated ketiminium ion (XI) is the proposed partitioning intermediate between the pathways.

reactions of the transaminases (Walsh, 1979), a pyridoxamine species is generated via a net 1,3 prototropic shift involving the C-α and C-4′ carbons of the substrate and cofactor, respectively (VIII → II → IX, Scheme II). Facile formation of a pyridoxamine–ketimine adduct (IX) effectively removes electron density from the α-carbon. The resulting protonated Schiff base serves to lower the energy barrier for β-proton removal, rendering this proton more acidic. The β-carbanion produced (X) then lends anchimeric assistance toward elimination of the γ-substituent, resulting in the formation of a β-γ unsaturated ketimine (XI). The protonated Schiff base renders this intermediate electron deficient and activates the γ-carbon toward Michael nucleophilic addition. Nucleophilic attack by the thiol group of cysteine and reversal of the previous steps result in a net γ-placement reaction (viz. VI to XVI).

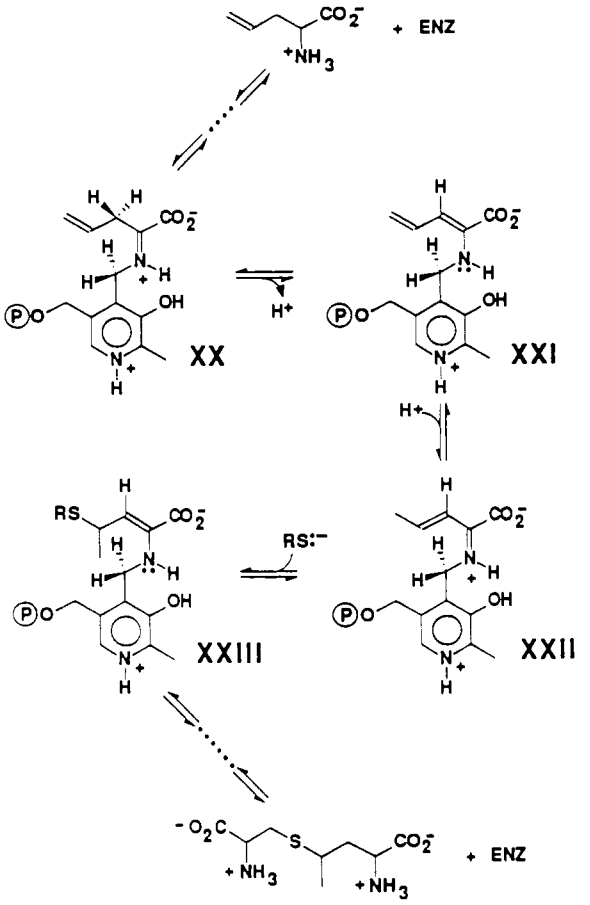
In the absence of an appropriate nucleophile, a tautomerization takes place that involves removal of the C-4′ proton, formation of the highly conjugated electron-rich vinylglycine quinonoid (IV), and subsequent protonation at the γ-carbon (V), giving an aminocrotonate species. Transaldimination involving the ε-amino group of a lysine side chain releases α-iminobutyric acid (Posner & Flain, 1972b) from the enzyme active site where it undergoes aqueous hydrolysis, yielding α-ketobutyrate and ammonia.

This mechanism provides the means for a plausible, effective labilization of the β-proton and proposes that the actual partitioning intermediate between the γ-replacement and γ-elimination pathways is the β-γ unsaturated α-iminopyridoxamine species (XI). This removes the paradox presented by Scheme I in which a single structural intermediate, the vinylglycine quinoid (IV), must display both electrophilic and nucleophilic character at the γ-carbon.

The reactivity of L-alanine and L-allylglycine is easily accommodated in this mechanistic picture (Scheme III). Analogous to the loss of lone-pair electrons by the elimination of a γ-substituent from OSHS, the electron lone pair is lost by protonation of the γ-carbon (XXII). In the presence of the appropriate nucleophile, the resulting β-γ unsaturated ketimine is susceptible to Michael addition at the γ-carbon and ultimately yields the new amino acid product γ-methylcystathionine. Since L-alanine is unable to lose the lone-pair electrons of the β-carbanion (either by elimination of a substituent or protonation), it is unable to proceed further along the reaction pathway. Since structures XX–XXIII all have tetrahedral geometries at the C-4′ carbon, the conjugation between the substrate and cofactor is effectively removed and these intermediates are likely to exhibit similar UV–visible absorption spectra arising from the pyridoxamine moiety of these intermediates. Comparison of the L-allylglycine and L-alanine reactions indicates that the increase in 300-nm absorbance is likely due to an equilibrium distribution of at least three intermediates.

The observation that L-allylglycine does not readily turn over to the corresponding α-keto acid, while OSHS and L-vinylglycine do, has interesting structural implications for the γ-replacement reaction. Disrupting the extended π-system of the α-carbanion (II) by protonation at the C-4′ carbon

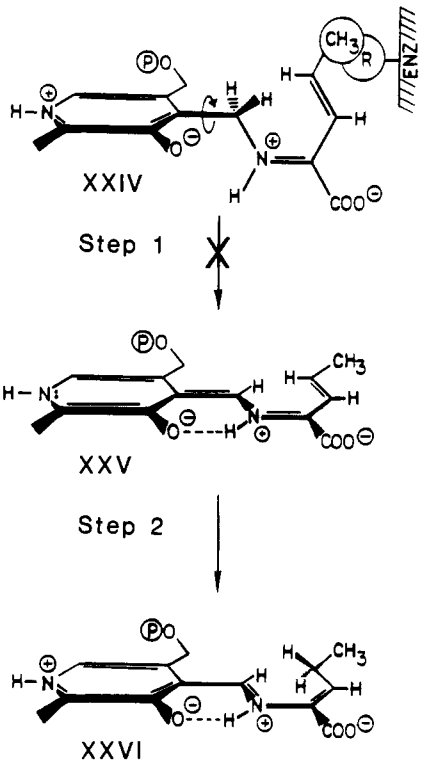
Scheme III: Proposed Reaction Mechanism for L-Allylglycine and the Formation of γ-Methylcystathionine



would allow rotation around either the C4–C4′ or C4–N σ-bonds and loss of planarity between the substrate and cofactor. Tautomerization to an aminocrotonate derivative, a species committed along the elimination pathway, would involve abstraction of a C-4′ proton, rotation to form a planar, highly conjugated structure, and facile protonation of the resulting electron-rich vinlyglycine quinonoid at the γ-carbon (Scheme II). OSHS and L-vinylglycine readily undergo this transformation. Substrates that have a γ-substituent, such as L-allylglycine, *trans*-4-methoxyvinylglycine (data not shown), and propargylglycine (Johnston, 1979b), do not readily form a 485-nm-absorbing intermediate. The inability of L-allylglycine to form an α-keto acid may result from steric restrictions at the active site imposed by the presence of a δ-carbon on the substrate. We speculate that the extra carbon inhibits formation of the planar structure (and the extended π-system) of the aminocrotonate derivative (Scheme IV, step 1). A similar situation arises from the reaction of cystathionine γ-synthase with *trans*-4-methoxyvinylglycine. This substrate produces spectral changes identical with those reported for the same reaction with methionine γ-lyase (Johnston et al., 1981). Despite the β-γ unsaturation, the presence of the 4-methoxy substituent prevents this compound from forming a highly conjugated, planar quinonoid, in marked contrast to vinylglycine itself. These observations suggest that the γ-replacement reaction occurs via interaction of the thiol nucleophile with the β-γ unsaturated ketiminium ion (XXIV, Scheme IV) in a conformation removed from planarity with the pyridine ring.

Review of the literature suggests that the formation of pyridoxamine intermediates is ubiquitous for PLP-dependent enzymes capable of generating β-carbanions. This process is

Scheme IV: Depiction of the β-γ Unsaturated Ketiminium Ion Removed from Planarity with the Pyridine Ring of the Cofactor (XXIV)[a]



[a] Steric restrictions may prevent the formation of a highly conjugated planar system (XXV), which would be necessary for protonation of the γ-carbon (XXVI).

observed in some transaminases that are known to undergo protonation at the C-4′ carbon of the cofactor (Walsh, 1979). Pyridoxamine intermediates have been proposed for reactions catalyzed by aspartate β-decarboxylase (Tate & Meister, 1974), kynureninase (Tanizawa & Soda, 1979), and γ-cystathionase (Washtien et al., 1977). All of these enzymes generate PLP-stabilized β-carbanions. In conclusion, herein we present the first direct evidence for such intermediates in the catalytic mechanism of cystathionine γ-synthase. We propose that the catalytic mechanisms of all PLP-dependent enzymes which catalyze the formation of β-carbanionic intermediates proceed via a pyridoxamine–ketiminium ion intermediate.

ACKNOWLEDGMENTS

We thank Dr. Melinda Roy for her assistance in the collection and analysis of the ^{1}H NMR data. We thank Dr. Edith Miles for her generous gift of *trans*-4-methoxyvinylglycine. We also thank Frank Moses and Kam Cheung for their invaluable assistance during the course of this study.

Registry No. OSHS, 1492-23-5; L-Cys, 52-90-4; L-Ala, 56-41-7; cystathionine γ-synthase, 9030-70-0; L-allylglycine, 16338-48-0; γ-methylcystathionine, 123933-45-9.

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Phase Equilibria of Cholesterol/Dipalmitoylphosphatidylcholine Mixtures: ^2H Nuclear Magnetic Resonance and Differential Scanning Calorimetry†

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ABSTRACT: Deuterium nuclear magnetic resonance spectroscopy and differential scanning calorimetry are used to map the phase boundaries of mixtures of cholesterol and chain-perdeuteriated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine at concentrations from 0 to 25 mol % cholesterol. Three distinct phases can be identified: the L_α or liquid-crystalline phase, the gel phase, and a high cholesterol concentration phase, which we call the β phase. The liquid-crystalline phase is characterized by highly flexible phospholipid chains with rapid axially symmetric reorientation; the gel phase has much more rigid lipid chains, and the motions are no longer axially symmetric on the ^2H NMR time scale; the β phase is characterized by highly ordered (rigid) chains and rapid axially symmetric reorientation. In addition, we identify three regions of two-phase coexistence. The first of these is a narrow L_α/gel-phase coexistence region lying between 0 and about 6 mol % cholesterol at temperatures just below the chain-melting transition of the pure phospholipid/water dispersions, at 37.75 °C. The dramatic changes in the ^2H NMR line shape which occur on passing through the phase transition are used to map out the boundaries of this narrow two-phase region. The boundaries of the second two-phase region are determined by ^2H NMR difference spectroscopy, one boundary lying near 7.5 mol % cholesterol and running from 37 down to at least 30 °C; the other boundary lies near 22 mol % cholesterol and covers the same temperature range. Within this region, the gel and β phases coexist. As the temperature is lowered below about 30 °C, the phospholipid motions reach the intermediate time scale regime of ^2H NMR so that spectral subtractions become difficult and unreliable. The third two-phase region lies above 37 °C, beginning at a eutectic point somewhere between 7.5 and 10 mol % cholesterol and ending at about 20 mol %. In this region, the L_α and β phases are in equilibrium. The boundaries for this region are inferred from differential scanning calorimetry traces, for the boundary between the L_α- and the two-phase region, and from a dramatic sharpening of the NMR peaks on crossing the boundary between the two-phase region and the β-phase region. In this region, the technique of difference spectroscopy fails, presumably because the diffusion rate in both the L_α- and β-phase domains is so rapid that phospholipid molecules exchange rapidly between domains on the experimental time scale.

Cholesterol is a major constituent of the plasma membrane of many of the cells of higher organisms, making up as much as 50 wt % of the lipid fraction in the case of the human erythrocyte membrane. Even so, its functional role within the membrane is not understood. The large changes which it induces in the physical properties of membranes suggest that part of its function may be to improve these characteristics over those of, for example, a simple phospholipid bilayer. Additionally, it may permit wider variations in composition of the other membrane constituents while maintaining the

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