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Conversion of the Aminocrotonate Intermediate Limits the Rate of γ-Elimination Reaction Catalyzed by L-Cystathionine γ-lyase of the Yeast *Saccharomyces cerevisiae*

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L-Cystathionine γ-lyase [EC 4.4.1.1] of *Saccharomyces cerevisiae* was shown to bind cofactor pyridoxal 5′-phosphate, up to 2 molecules/subunit. The association constants of the enzyme for the cofactor were estimated to be 3.67×10^5 M^{-1} and 9.05×10^3 M^{-1}. However, the latter value was too small for the binding to play a catalytic role. Changes in the absorption spectra of the enzyme in γ-elimination reaction mixtures with various amino acids as substrates were observed at 10°C to elucidate the reaction mechanism of the enzyme. The enzyme formed a chromophore exhibiting absorption at approximately 480 nm, which is characteristic of an aminocrotonate intermediate with *O*-succinyl-L-homoserine, L-cystathionine, L-homoserine, or *O*-acetyl-L-homoserine, at rates in this order. The intermediate was consumed at much lower rates than those of formation. The order of the rates of consumption was the same as the order of the formation rates and the order of the γ-elimination activity of the enzyme with the above-mentioned substrates. These results strongly suggested that the intermediate was essential for γ-elimination and that the reaction was rate-limited by its conversion into the product α-ketobutyrate. L-Cysteine sensitively inhibited the α, γ-elimination activity of the enzyme, and also retarded the formation of the chromophore when it was provided to the enzyme together with a substrate. The reason for these phenomena is discussed.

Key word: aminocrotonate intermediate, cystathionine γ-lyase of *S. cerevisiae*, pyridoxal-5′-phosphate, thiazolidine.

Abbreviations: CTT, L-cystathionine; PLP, pyridoxal 5′-phosphate; K-P, pottasium phosphate.

L-Cystathionine (CTT) γ-lyase [EC 4.4.1.1] of *Saccharomyces cerevisiae* catalyzes various reactions, including α, β-elimination, α, γ-elimination, and γ-replacement (*1*). Among them, the second reaction with CTT as the substrate plays a role in the synthesis of cysteine (*2*). The product L-cysteine inhibits the enzyme, and at the same time the amino acid can also be a substrate for the enzyme in the β-elimination reaction (*1*). Furthermore, cysteine reacts with the aldehyde group of the cofactor of the enzyme pyridoxal 5′-phosphate (PLP) to form thiazolidine (*3*). We recently characterized the β-elimination activity of the enzyme with L-cysteine and L-cystine as substrates, and showed that the inhibition by cysteine is due mainly to removal of PLP (*4*). Continuous observation of the absorption spectrum of the enzyme has revealed that the absorption at 420 nm characteristic of an internal Schiff base formed with the enzyme and PLP is reduced to approximately 10% when a sufficient amount of L-cysteine is added, and that the new absorption characteristic of an external Schiff base with cysteine is seen only after an excess amount of cysteine is consumed.

Some interesting information concerning interactions between amino acids and PLP, the cofactor of CTT-metabolizing enzymes, has been presented. *Streptomyces phaeochromogenes* CTT γ-lyase has been reported to have two sites for cysteine binding; one is the active site where cysteine is converted into pyruvate and the other is the regulatory site to which cysteine specifically binds to inhibit the catalytic activity (*5*). Rat liver CTT γ-lyase has two nonequivalent binding sites for PLP; one binds a 4C compound (L-homoserine) and the other reacts with a 3C compound (L-cysteine) (*6–8*). The same enzyme of *Neurospora crassa* has been reported to bind up to 8 PLP/tetrameric enzyme, only 4 of which are functional as the cofactor (*9*). CTT β-synthase of *S. cerevisiae*, which synthesizes CTT with homocysteine and serine, was recently reported to be rate-limited by conversion of an aminoacrylate intermediate (*10*). In order to elucidate the reaction mechanism of *S. cerevisiae* CTT γ-lyase, the binding of PLP to the enzyme and spectrophotometric behavior of the bound PLP in the γ-elimination reaction were studied.

In this study, we found that the enzyme of *S. cerevisiae* bound PLP, up to 2/subunit, but only one of them, the one that exhibited higher affinity, was functional as the cofactor in the γ-elimination reaction, and that an aminocrotonate intermediate with a substrate amino acid was essential for the catalysis, the consumption of which was rate-limiting.

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MATERIALS AND METHODS

Materials—*O*-Acetyl-L-homoserine was synthesized as described previously (*1*). CTT and *O*-succinyl-L-homoserine (Sigma Chemicals, St. Louis, MO), L-cysteine hydrochloride (Kishida Chemicals, Osaka), PLP and L-homoserine (Nacalai Tesque, Kyoto), and other chemicals were obtained commercially and were of the highest grade available. CTT γ-lyase (*1*) and *O*-acetyl-L-serine·*O*-acetyl-L-homoserine sulfhydrylase [EC 4.2.99.10] (*11*) of *S. cerevisiae* were both purified from transformed *Escherichia coli* cells as described previously.

Preparation of the Holoenzyme—A purified preparation of the enzyme (7 mg of protein/ml) was dialyzed overnight against a sufficient volume of 0.1 M potassium phosphate (K-P) buffer (pH 7.8) containing 1 mM EDTA and 0.5 mM PLP. The dialysis medium was changed and dialysis was continued for a further 5 h, and then the dialyzate was centrifuged for 20 min at 12,000 × *g*. The supernatant fraction obtained was used for PLP-binding analysis and observation of absorption spectra.

Determination of Thiazolidine Formed with the Holoenzyme and L-Cysteine—After obtaining an absorption spectrum in the wavelength range of 300–500 nm for a solution of the holoenzyme dissolved in 0.1 M K-P buffer (pH 7.8) containing 1 mM EDTA (0.022 mM for the subunit) at 10°C against the same buffer, the sample was mixed with L-cysteine hydrochloride at the concentration of 2.5 mM. Immediately after this, the increase in A_{330} characteristic of thiazolidine was observed until it reached a plateau. The dialysis buffer employed to prepare the holoenzyme was also subjected to the same procedure as a reference. The amount of thiazolidine formed was calculated, on the basis of A_{330} obtained for freshly prepared PLP solutions (20 μM and 50 μM).

Equilibrium Dialysis of the Enzyme—A purified preparation of the enzyme was appropriately diluted with 0.1 M K-P buffer (pH 7.8) containing 1 mM EDTA to give 0.5 and 1 mg of protein/ml. Aliquots (2 ml each) of the two solutions were subjected to dialysis at 4°C against 300 ml of the same buffer as that described above, which contained eleven different concentrations of PLP (0, 0.005, 0.007, 0.01, 0.015, 0.02, 0.05, 0.08, 0.11, 0.15, and 0.2 mM). The dialysis was continued for three days with one change of the dialysis buffer. As a control, a purified preparation of *S. cerevisiae O*-acetyl-L-serine·*O*-acetyl-L-homoserine sulfhydrylase (*11*) was also subjected to dialysis in the same way at the protein concentration of 1 mg/ml. The concentrations of PLP in the dialyzates and dialysis buffers were determined with 2,4-dinitrophenylhydrazine by the method of Friedmann and Haugen (*12*).

Observation of Changes in the Absorption Spectra of the Enzyme with Various Substrate Amino Acids—To a solution (1.425 ml) comprising the holoenzyme [at the concentration of 1.68 mg/ml, 0.035 mM for the subunit (*1*)], 0.1 M K-P buffer (pH 7.8), 1 mM EDTA, and 0.2 mM PLP, 0.075 ml of a 50 mM substrate amino acid solution was added to 2.5 mM. Absorption spectra were determined at 10°C with a spectrophotometer (Hitachi Model 124) in the wavelength range of 350–550 nm (in 100 seconds) at the times (min) indicated in Fig. 1 with the same solution but lacking the enzyme as a reference. Increases in the absorption at 480 nm were observed at 5°C in order to compare the rates of aminocrotonate formation with various substrates, since the formation was so rapid at 10°C that exact comparison of the rates was difficult.

Determination of the α, γ-Elimination Activity of the Enzyme with CTT and L-Homoserine as Substrates—Reactions were carried out at 30°C for 30 min in 1 ml reaction mixtures comprising 0.1 M K-P buffer (pH 8.0) and 0.2 mM PLP with 5 mM CTT and L-homoserine as substrates, using 0.022 mg of the purified CTT γ-lyase. α-Ketobutyric acid produced from these substrates was assayed using lactic dehydrogenase [EC 1.1.1.27] and NADH, as described previously (*4*).

Protein Concentrations—Protein concentrations were determined by the methods of Lowry *et al.* (*13*), and Bradford (*14*).

RESULTS

The Amount of Thiazolidine Formed with the Enzyme and L-Cysteine—In order to determine the amount of PLP bound to the enzyme, the amount of thiazolidine formed with the holoenzyme and cysteine was photometrically determined as described under "MATERIALS AND METHODS." After obtaining absorption spectra of the solutions of holoenzyme and the dialysis buffer, both in the presence of cysteine, the difference in A_{330} (due to thiazolidine) observed for the former was corrected for that due to free PLP contained in the sample, on the assumption that the concentration of PLP was the same as that in the latter. Net absorption due to the protein-bound PLP was compared with that for the standard PLP solutions. Three determinations were carried out and an average value of 2.1 mol of thiazolidine/mol of subunit was obtained.

Scatchard Plot Analysis of PLP Binding to the Enzyme—In order to ascertain the number of PLP bound to the subunit and to determine the association constant, equilibrium dialysis of the enzyme was carried out against buffers containing PLP at various concentrations, as described under "MATERIALS AND METHODS." The concentrations of PLP were determined for both the dialyzates and dialysis buffers, and the amounts of PLP that bound to the protein were calculated as the differences between the two values. The protein concentrations of the dialyzates were also determined. The ratios of r (bound PLP/subunit of the enzyme) to free PLP were plotted against r, which was calculated on the basis of a subunit molecular weight of 48,000 (*1*) (data not shown). From the Scatchard plot, it can be seen that the enzyme bound up to 2 PLP/subunit. The results obtained for *O*-acetyl-L-serine·*O*-acetyl-L-homoserine sulfhydrylase (*15*) of the same organism that had been subjected to dialysis in the same way gave a maximal value of 1 for *r* (PLP/subunit). Based on the assumption that the enzyme subunit has two independent binding sites for PLP, *r* is expressed as follows (*16*):

$$r = K_1L/(1+K_1L) + K_2L/(1 + K_2L),$$

where L is the concentration of free PLP. For two arbitrarily chosen points, the values of r and L were read. By using these values, simultaneous equations were obtained, and association constants K_1 and K_2 were calcu-

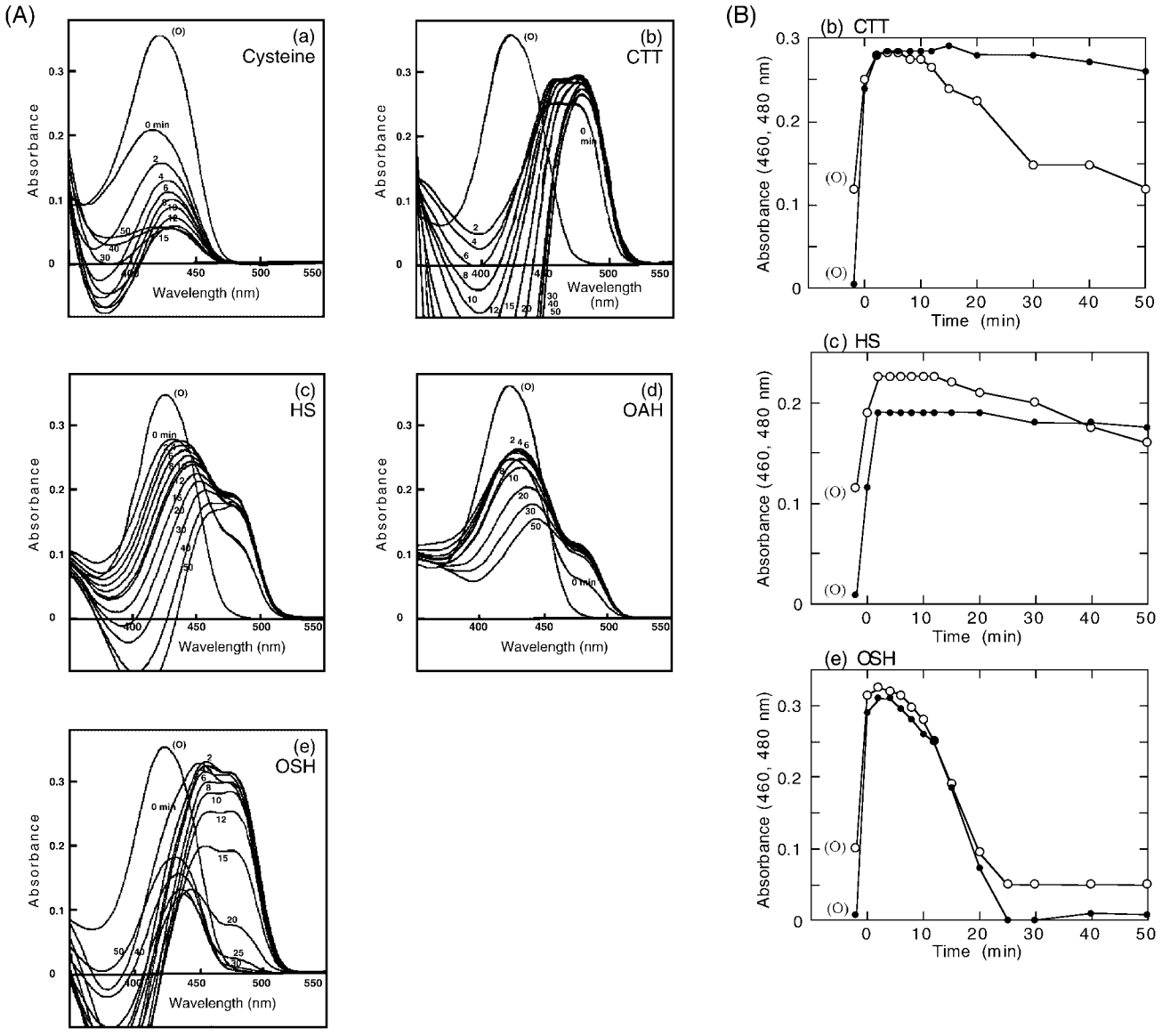

Fig. 1. **(A) Changes in the absorption spectra of the enzyme during elimination reactions with various amino acids as substrates.** Absorption spectra of the enzyme (0.035 mM for the subunit) were determined with various amino acids as substrates (each 2.5 mM) for the elimination reaction carried out at 10°C, as described under "MATERIALS AND METHODS." The numbers on curves represent the times (in min) after mixing with substrate solutions, at which the spectra were obtained. Symbols: (O), without a substrate; HS, L-homoserine; OAH, *O*-acetyl-L-homoserine; OSH, *O*-succinyl-L-homoserine. **(B) Time courses of the absorption at 460 nm (open circles) and 480 nm (closed circles).** The absorption values were read from the spectra for the mixtures with CTT (b), HS (c), and OSH (e), and plotted against the incubation time. The values for the holoenzyme solutions are shown by (O).

lated using these equations to be 3.67×10^5 M^{-1} and 9.05×10^3 M^{-1}, respectively.

Spectral Changes of the Enzyme with Amino Acid Substrates—In order to investigate the behavior of the enzyme-bound PLP in the presence of a substrate amino acid, the change in the absorption spectrum of the enzyme was observed at 10°C during the reaction, as described under "MATERIALS AND METHODS." The low temperature (10°C) used in this experiment permitted more exact observation of the spectral change than in the case of 30°C. The results obtained with L-cysteine (a), CTT (b), L-homoserine (c), *O*-acetyl-L-homoserine (d), and *O*-succinyl-L-homoserine (e) employed as substrates for the elimination reaction are shown in Fig. 1A. The holoenzyme solution gave a spectrum exhibiting an absorption at around 420 nm, which is characteristic of an internal Schiff base formed with PLP and an ε-amino group of a lysine residue of the enzyme (O).

The spectral change with L-cysteine indicated that the Schiff base of the enzyme decreased to approximately 14% of that of the holoenzyme solution, which was almost the same ratio as observed at 30°C (*4*). However, spectra (b), (c), and (e) showed the formation of two chromophores exhibiting absorption at around 460 nm and 480 nm immediately after the addition of the substrates, but spectrum (d) with acetylhomoserine gave an unclear result with respect to A_{460}. When CTT (b), homoserine (c), and acetylhomoserine (d) were employed as the sub-

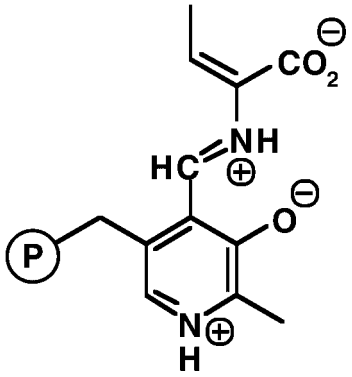

Fig. 2. **Structure of the aminocrotonate intermediate.**

strates, the level of the A_{480} chromophore attained seemed to be maintained at an almost constant level for a certain period. However, the chromophore decreased very rapidly in the case of succinylhomoserine (the best substrate for this enzyme) (e). The level of another chromophore (A_{460}) decreased more rapidly than that of the A_{480} chromophore in the mixtures of the two amino acids [(b) and (c)], but the levels of both chromophores seemed to decrease in parallel in the mixture with succinylhomoserine (e). The changes in the absorption of the two chromophores, as described above, were read from the spectra and plotted against incubation time (Fig. 1B). It is more evident from the figure that the differences in the absorption behavior are related to the differences in the catalytic activities of the enzyme with these amino acids as substrates (*1*). It was, therefore, concluded that the enzyme also formed an aminocrotonate intermediate (Fig. 2) that absorbed light at approximately 480 nm with substrates with the 4C skeleton, as other amino acid-metabolizing enzymes do (*17–19*).

Velocities of Formation of Aminocrotonate Intermediates with Various Amino Acids as Substrates—The increase in the absorption at around 480 nm was so rapid at 10°C that it was difficult to exactly compare the velocities with the four amino acid substrates (Fig. 1). In order to make the differences in the velocities more distinct, changes in the absorbance at 480 nm were observed at 5°C for the same reaction mixtures as those described above. The absorbance behavior with these amino acids is summarized in Fig. 3A, from which the initial velocities of the increase in the absorbance were calculated, and are shown in Table 1A.

Velocities of Consumption of Aminocrotonate Intermediates Formed with Various Amino Acids—It was evident that the decrease in the absorption at around 480 nm was much slower than the increase (Fig. 1). Therefore, the decrease was observed at 30°C for the same reaction mixtures (Fig. 3B). The velocity of the consumption of the chromophore was calculated by the decrease in the absorption in a region showing a linear decrease, as indicated in the figure. The results obtained are summarized in Table 1B. It should be noted that the absolute values shown in (B) are much smaller than those in (A), even though the former observation (B) was performed at 30°C and the latter (A) at 5°C. It also became evident that the order of the rates of change in A_{480} in both directions (formation and consumption of the chromophore) was the same as that of the γ-elimination activity with succinylhomoserine, CTT, homoserine, and acetylhomoserine (*1*).

Effects of L-Cysteine on the α, γ-Elimination Activity of the Enzyme and Formation of an Aminocrotonate Intermediate—In order to confirm the inhibitory effect of cysteine on CTT α, γ-elimination activity, elimination reactions were carried out as described under "MATERIALS AND METHODS" with 5 mM CTT and L-homoserine in the presence of L-cysteine at various concentrations, as indicated in Fig. 4. The results with CTT as a substrate are shown by open circles. The activity of the enzyme was 0.63 μmol/min/mg of protein in the absence of cysteine, but the activity level decreased markedly to approximately 30% with an increase in the cysteine concentration up to around 1.5–2.0 mM. When CTT was replaced by L-homoserine, very similar results were obtained (closed circles).

As the significance of the aminocrotonate intermediate became clear, we attempted to observe the behavior of the intermediate in the presence of cysteine. The time courses of the absorption at 480 nm were observed for reaction mixtures with L-homoserine as the substrate, with various concentrations of cysteine. The results obtained are summarized in Fig. 5. It is evident that com-

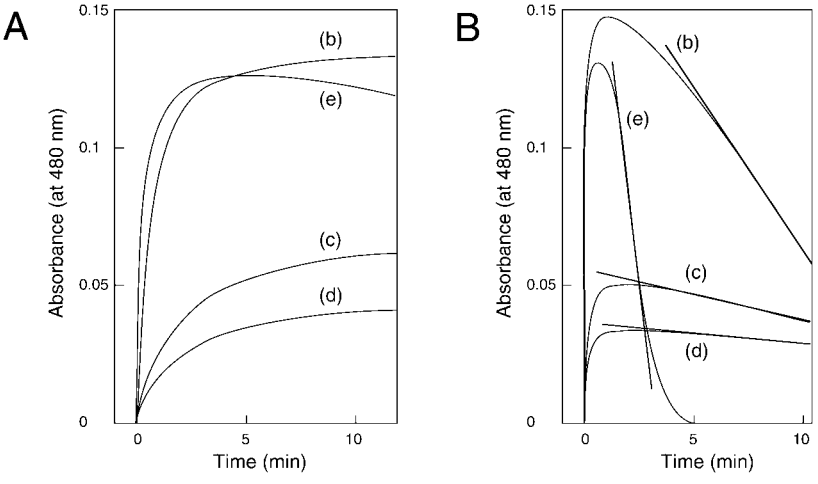

Fig. 3. **Behavior of the absorbance at 480 nm in reaction mixtures with various amino acids as substrates.** (A) The reaction mixtures containing the holoenzyme (approximately 0.017 mM subunit) were kept at 5°C. Immediately after mixing with the solution of a substrate amino acid at a final concentration of 2.5 mM, the change in the absorption at 480 nm was recorded. Other conditions were the same as in Fig. 1. The initial increase in the absorption was calculated. (B) Reactions were carried out in the same way as above, but at 30°C, and the absorption changes were recorded. The decrease in the absorption was calculated in the region shown by a straight line on each curve. The amino acids employed were CTT (b), L-homoserine (c), *O*-acetyl-L-homoserine (d), and *O*-succinyl-L-homoserine (e).

Table 1. **Comparison of the velocities of formation and consumption of the aminocrotonate intermediate with various amino acids as substrates.**

Amino acids	Increase in A_{480}/min (5°C) (A)	Decrease in A_{480}/min (30°C) (B)
CTT	0.11	–0.013
L-Homoserine	0.023	–0.002
O-Acetyl-L-homoserine	0.013	–0.001
O-Succinyl-L-homoserine	0.26	–0.064

Values were read from Fig. 3.

plete formation of the aminocrotonate intermediate was prevented when the concentration of cysteine added to the reaction mixture was increased to 10 mM. However, its consumption appeared not to be greatly affected by cysteine.

DISCUSSION

In order to elucidate the reaction mechanism of CTT γ-lyase of *S. cerevisiae*, we attempted to accurately determine the number of PLP that bound to the subunit and to analyze the behavior of PLP as a cofactor during the γ-elimination reaction. Binding of PLP to the enzyme was investigated in two ways, as described under RESULTS. The results suggested that the enzyme had two different sites (per subunit) with different affinities to PLP, the association constants being 3.67×10^5 M^{-1} and 9.05×10^3 M^{-1}. The latter value is very small compared with the values reported for other PLP-binding enzymes (*21*), and the site therefore seems not to be functional under physiological conditions. Based on the results obtained, the *S. cerevisiae* enzyme is unlikely to have nonidentical PLP-binding sites that are both functional, as described for other enzymes (*6–8*). Similar results have been reported for the *Neurospora* CTT γ-lyase (*9*).

For as exact comparison of spectral changes of the enzyme as possible, absorption spectra were determined at 10°C rather than 30°C, on the assumption that no significant change in the reaction mechanism occurs (Fig. 1). Although four amino acids other than cysteine caused drastic changes in the spectra after being dissolved in the enzyme solution, cysteine just caused a simple decrease in the absorption accompanying a slight red shift of the peak with time, the change being essentially the same as that reported previously (*4*). The spectral change common to the four samples (with 4C skeleton amino acids) was the appearance of two new absorption bands at around 460 nm and 480 nm, although the former was unclear with acetylhomoserine (d). The latter is due to the formation of an aminocrotonate intermediate (Fig. 2) with each amino acid, as has been reported for other enzymes (*17–19*). In spectra (b)–(d), A_{480} slowly decreased with time during the reaction, after remaining unchanged for certain periods because of abundant amounts of substrates compared with the poor reactivities of the enzyme with these substrates (*1*). However, in the case of (e) (for succinylhomoserine), it decreased immediately after the beginning of the reaction. The different velocities of consumption of the aminocrotonate intermediate were more exactly observed in a separate experiment (Fig. 3B), and quantitatively presented in Table 1 (B). The different behaviors of the chromophore can be well explained, from the standpoint that it is the intermediate, by the enzyme's different reactivities to these subsrates: the enzyme best reacts with succinylhomoserine (*1*). The rates of formation of the chromophore (A_{480}) (Table 1A) were much faster than the rates of consumption (B) observed for all amino acids. Based on these

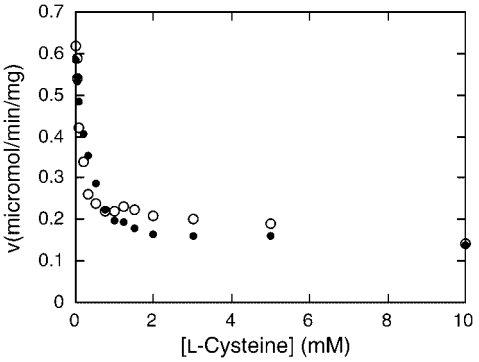

Fig. 4. **Inhibition of the α, γ-elimination activity of *S. cerevisiae* CTT γ-lyase by L-cysteine.** The α, γ-elimination reaction was carried out as described under "MATERIALS AND METHODS" using 0.022 mg of the enzyme protein with 5 mM CTT (open circles) or 5 mM L-homoserine (closed circles) as the substrate, in the presence of the indicated concentrations of cysteine. The amounts of the reaction product, α-ketobutyric acid, were determined by monitoring the oxidation of NADH in the presence of lactic dehydrogenase [EC 1.1.1.27]. Other conditions are given under "MATERIALS AND METHODS."

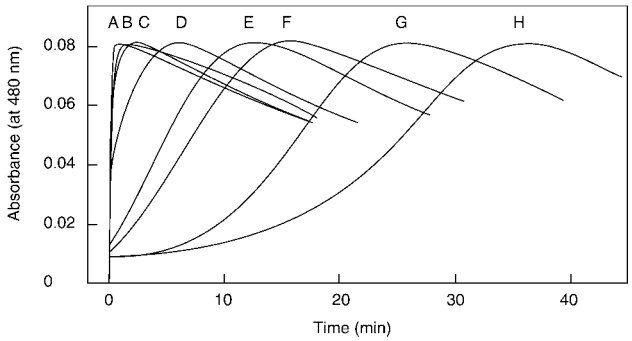

Fig. 5. **Formation of an aminocrotonate intermediate in the γ-elimination reaction with L-homoserine as the substrate in the presence of L-cysteine at various concentrations.** L-Homoserine was added to the reaction mixture to a final concentration of 5 mM, together with various amounts of L-cysteine. The holoenzyme was employed at a concentration of approximately 0.02 mM (for the subunit). The final concentrations of L-cysteine were as follows (in mM): (A) 0.1, (B) 0.25, (C) 0.5, (D) 1.0, (E) 2.0, (F) 2.5, (G) 5.0, and (H) 10.0. Other conditions were the same as those in the experiments in Fig. 1. The solutions were incubated at 30°C.

results, it was concluded that aminocrotonate is an essential intermediate in the γ-elimination reaction catalyzed by this enzyme and that the rate-limiting step is conversion of this intermediate into the product. This is in contrast to the case for *O*-acetylserine sulfhydrylase reaction, in which the formation of the aminoacrylate intermediate is rate-limiting (*22*).

The rates of disappearance of A_{460} were faster than those of A_{480} in (b) and (c) (Fig. 1B). In the case of *O*-succinylhomoserine (e), the rates of disappearance of the two absorption bands (A_{460} and A_{480}) were almost the same. This can be attributed to the very high rate of conversion of the aminocrotonate intermediate (A_{480}) into the product α-ketobutyrate. These observations suggest that the A_{460} chromophore is also involved in some way in the catalytic activity of this enzyme. Similar behavior of a chromophore exhibiting absorption at around 460 nm has been observed under steady-state conditions for CTT γ-synthase of *Salmonella* in the β-elimination of *O*-succinyl-L-serine (*17*) and β-haloaminobutyrate (*18*). CTT β-synthase of *S. cerevisiae* has been reported to produce a similar chromophore (A_{459}) under pre-steady-state conditions for the β-replacement reaction (*10*). This information suggests that the A_{460} chromophore is involved in the metabolism of β-carbon. However, determination of its role in γ-elimination with 4C-substrates catalyzed by *S. cerevisiae* CTT γ-lyase has to wait further investigation.

It has been reported that cysteine, the product of the CTT γ-elimination reaction, inhibits the reaction in mammals (*20*) and also in the yeast *S. cerevisiae* (*1*, *2*). In this study, the inhibitory effects of cysteine on γ-elimination reactions with CTT and homoserine as substrates (Fig. 4), and the formation of the aminocrotonate intermediate (Fig. 5) were ascertained by more detailed experiments. We have speculated that cysteine provived as the substrate for the β-elimination reaction inhibits the enzyme of *S. cerevisiae* by removing PLP from the holoenzyme when the concentration of cysteine is higher than a certain level (*4*). In the case of γ-elimination also, the possibility that cysteine partially removed PLP from the holoenzyme to form thiazolidine can not be excluded. However, it was recently demonstrated that the enzyme catalyzes the CTT γ-synthase reaction with homoserine and cysteine as substrates at a comparative or slightly higher rate to that of homoserine γ-elimination (Akamatsu *et al.*, unpublished data). Therefore, it must be considered that the inhibition of γ-elimination and retardation of the chromophore formation mentioned above are result of being largely affected by the γ-replacement reaction occurring with cysteine as the substrate. The inhibition shown in Fig. 4 is considered to have been caused by the involvement of a large amount of active enzyme in the γ-replacement reaction. This consideration is supported by the fact that *Salmonella* CTT γ-synthase also catalyzes γ-elimination of succinylhomoserine in the absence of cysteine, but the reaction does not begin in the presence of cysteine until it is completely consumed by the γ-replacement reaction (*17*). The formation of the aminocrotonate intermediate occurred after certain periods depending on the amount of cysteine added (Fig. 5). The time of retardation almost corresponded to the time of consumption of cysteine as a substrate for γ-replacement, and not to the time of cysteine decomposition through the β-elimination activity. This activity [approximately 0.1 μmol/min/mg (*4*)] is too low to decompose cysteine added in the retardation time shown in Fig. 5.

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