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CYSTATHIONINE γ-SYNTHASE: STUDIES ON THE γ REPLACEMENT REACTION

BARRY I. POSNER*

Laboratory of Biochemistry, Section on Enzymes, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

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SUMMARY

Cystathionine γ-synthase from *Salmonella* catalyzes the γ replacement reaction in which *O*-succinyl-L-homoserine and L-cysteine form succinic acid and cystathionine, an important intermediate in methionine biosynthesis. In the absence of L-cysteine the enzyme catalyzes a γ elimination reaction in which *O*-succinylhomoserine is decomposed to α-ketobutyrate, NH_3 and succinic acid. β-Mercaptopropionic acid, a noncompetitive inhibitor of γ elimination, has been shown to be a substrate for the γ replacement reaction. The reaction product has been identified, by mass spectroscopy, as *S*-carboxyethyl-L-homocysteine. A kinetic study of this γ replacement reaction has demonstrated that β-mercaptopropionic acid has no effect on the enzyme's affinity for *O*-succinylhomoserine. It is suggested that the γ replacement reaction with L-cysteine is kinetically similar to that with β-mercaptopropionic acid.

INTRODUCTION

Cystathionine γ-synthase, isolated from *Salmonella*[1] is unique among known pyridoxal phosphate enzymes in catalyzing the reactions of γ replacement and γ elimination[2]. The former reaction (Reaction 1) is a key step in methionine biosynthesis, while the latter (Reaction 2) occurs in the absence of cysteine.

O-Succinyl-L-homoserine + L-cysteine → L-cystathionine + succinic acid (1)

O-Succinyl-L-homoserine + H_2O → α-ketobutyrate + succinic acid + NH_3 (2)

In this investigation we demonstrate that β-mercaptopropionate, previously shown to be a noncompetitive inhibitor of Reaction 2 (ref. 2), is a substrate in the γ replacement reaction. A kinetic analysis of this reaction indicated that β-mercaptopropionate does not alter the affinity of the enzyme for *O*-succinylhomoserine. This suggests that the γ replacement reaction is kinetically identical to the β replacement reaction[3].

* Present address: McGill University Clinic, Royal Victoria Hospital, Montreal, Canada.

MATERIALS

Cystathionine γ-synthase was prepared by a modification of the original procedure[4]. *O*-Succinylhomoserine was synthesized as previously described[5].

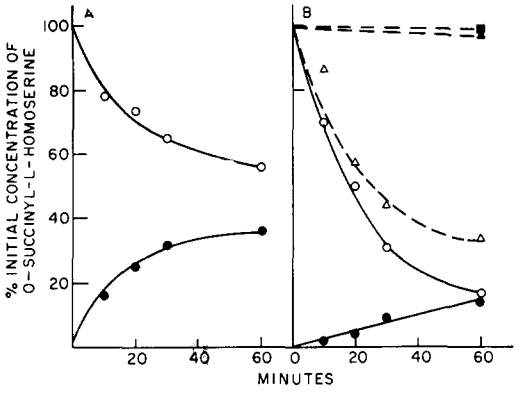


Fig. 1. Time course of cystathionine synthase-catalyzed formation of α-ketobutyrate and disappearance of *O*-succinyl-L-homoserine in the absence (A) and in the presence (B) of β-mercaptopropionate. The reaction mixtures contained, in 2 ml final volume, 200 μmoles of potassium pyrophosphate, pH 7.5; and 17 μmoles of *O*-succinyl-L-homoserine. β-Mercaptopropionate was present at 10 μmoles/ml and its concentration has been normalized to that of *O*-succinyl-L-homoserine. After the solution was brought to 35 °C, nitrogen was bubbled through it for 30 s and over its surface for 10 min, and the reaction was started by adding 20 μg (0.26 unit) of cystathionine synthase. At the indicated times, 0.2-ml aliquots were pipetted into 1.8 ml of 0.2 M cold trichloroacetic acid. The solution was then brought to pH 6.5 with 10 M KOH. The subsequent determination of α-ketobutyrate, *O*-succinylhomoserine, and β-mercaptopropionate are noted in the text. ●, α-ketobutyrate; ○, *O*-succinyl-L-homoserine; β-mercaptopropionate, △, in complete reaction mixture; ■, in mixture without *O*-succinyl-L-homoserine; ▼, in mixture without enzyme.

RESULTS AND DISCUSSION

Fig. 1 shows the change in concentration of *O*-succinyl-L-homoserine and α-ketobutyrate during the course of the γ elimination reaction in the presence and absence of β-mercaptopropionic acid. *O*-Succinyl-L-homoserine was measured in the presence of α-ketobutyrate in the following manner. The reaction mixture contained, in a 1-ml volume, 100 μmoles of potassium pyrophosphate (K and K laboratories, Plainview), pH 8.2; 9.25 μmoles of NADH (Sigma); and aliquots of the reaction mixture (legend to Fig. 1) containing 0.05–0.2 μmole of *O*-succinyl-L-homoserine. After an initial reading of the absorbance at 340 nm, 200 μg of lactic dehydrogenase (Boehringer) was added and the change in absorbance was noted. This allowed for a determination of the amount of α-ketobutyrate present in the sample. When the new level of absorbance was attained 20 μg of cystathionine synthase was added. The subsequent total change in absorbance (molar absorbance = 6200) corresponded to the amount of *O*-succinyl-L-homoserine which had been present and converted to α-ketobutyrate*.

* *O*-Succinylhomoserine could be measured in the presence of β-mercaptopropionic acid since the concentration of β-mercaptopropionic acid in the final assay mixture never exceeded 0.1 μmole/ml at which concentration its effect on γ elimination is negligible.

In the absence of β-mercaptopropionate (Fig. 1A) the disappearance of *O*-succinyl-L-homoserine is accompanied by the appearance of an approximately equal amount of α-ketobutyrate. In the presence of β-mercaptopropionate (Fig. 1B) there is an increase in the rate of disappearance of *O*-succinyl-L-homoserine accompanied by an inhibition of both the initial rate and the extent of α-ketobutyrate formation. The concentration of β-mercaptopropionate as measured with the sulfhydryl reagent, 5,5′-dithiobis-(2-nitrobenzoic acid)[6], remains unchanged, over 60 min of incubation, in the absence of either *O*-succinyl-L-homoserine or enzyme; but declines in the complete reaction mixture. The amount of β-mercaptopropionate which disappeared approximately equals the *O*-succinyl-L-homoserine which disappeared but was not converted to α-ketobutyrate.

When the reaction mixture was acidified and passed over Dowex 50 (H^+) (Calbiochem), a ninhydrin positive material was eluted from the column with 1 M NH_4OH. Since this procedure converts *O*-succinylhomoserine to the ninhydrin-negative compound *N*-succinylhomoserine, by means of an O → N acyl shift, the above observation indicated that a new amino acid was formed during the course of the reaction.

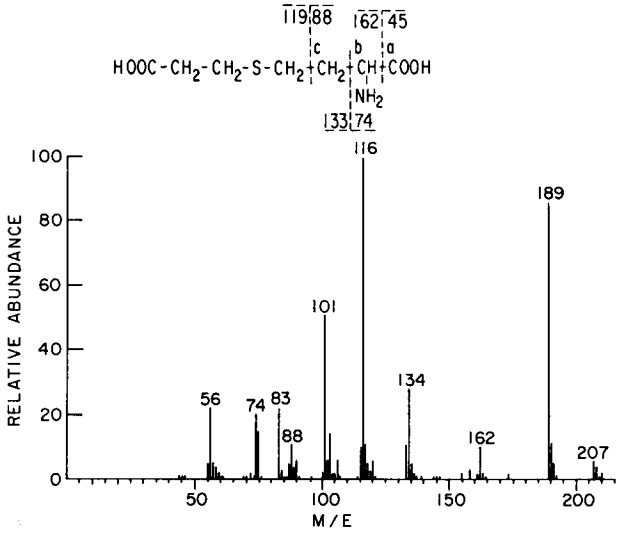
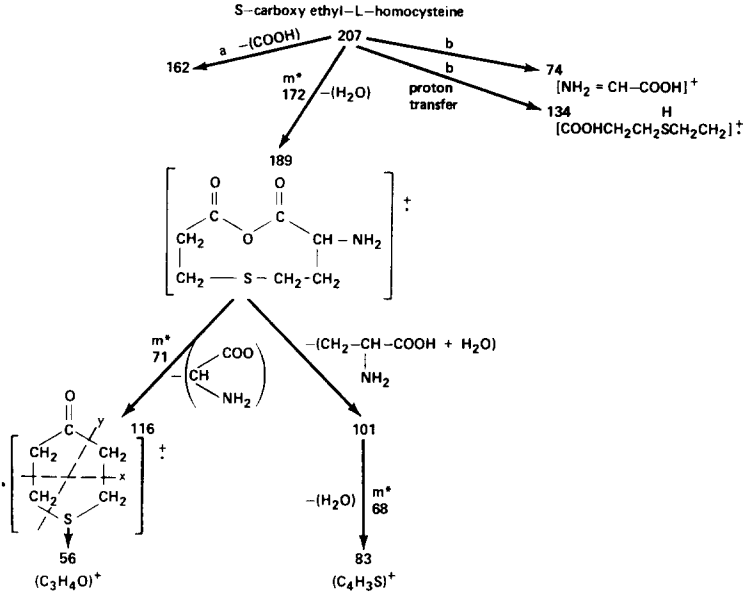


Fig. 2. Mass spectrum of the amino acid-product of the reaction, depicted in Fig. 1B, obtained with 20-V electrons. The sample, lyophilized after elution from Dowex 50, was introduced by direct inlet into the LKB mass spectrometer (Type 9000). The spectrum was taken at an inlet temperature (externally heated) of 150–160 °C, after a more volatile hydrocarbon contaminant had come off at 90–100 °C. The ionizing current was 60 μA.

The mass spectrum of the material obtained from the Dowex 50 eluate is compatible with *S*-carboxyethyl-L-homocysteine (Fig. 2). Scheme 1 outlines reaction pathways which yield the fragmentation pattern seen in Fig. 2. Metastable peaks have not been shown in Fig. 2 but are noted in Scheme 1. A metastable peak at *m/e* 172 indicates that the ion at *m/e* 189 arises by loss of H_2O from *m/e* 207. This suggests that the ions > *m/e* 207 are due to contaminants and that *m/e* 207 corresponds to the parent peak with the predicted molecular weight for *S*-carboxyethyl-L-homocysteine.



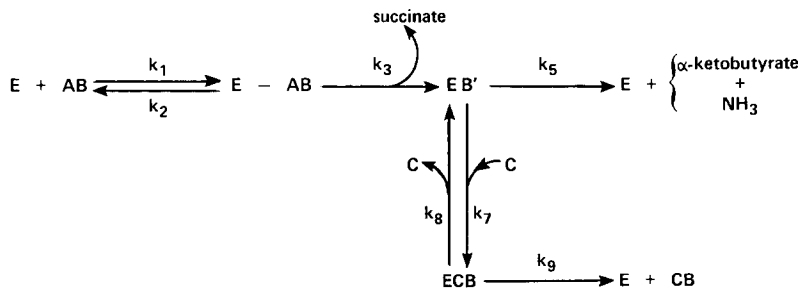
Scheme 1. Reaction pathways for fragment formation in the mass spectrometer. a and b refer to cleavage sites depicted in Fig. 2. m* refers to metastable peaks.

The relatively large peak at *m/e* 191 is consistent with there being a sulfur atom in the molecule. Since *m/e* 189 does not undergo further decarboxylation we suggest that this ion is the anhydride of the dicarboxylic acid. We have no proof that *m/e* 101 arises as depicted from *m/e* 189 but this seemed more plausible than its arising from the other larger ions.

S-Carboxyethyl-L-homocysteine would be formed during the course of the incubation by the following reaction:

$$\beta\text{-mercaptopropionic acid} + \textit{O}\text{-succinyl-L-homoserine} \rightarrow \textit{S}\text{-carboxyethyl-L-homocysteine} + \text{succinic acid} \qquad (3)$$

Scheme 2 is a proposed reaction sequence for cystathionine synthase-catalyzed γ elimination (Sequence k_1, k_3, k_5), and γ replacement (Sequence k_1, k_3, k_7, k_9) in the



Scheme 2. Reaction sequence for cystathionine synthase-catalyzed γ elimination and γ replacement reactions. See text for description.

absence and presence, respectively, of β-mercaptopropionate. In this scheme E is cystathionine synthase; AB, *O*-succinyl-L-homoserine; E–AB, the complex of enzyme and *O*-succinyl-L-homoserine; EB′, enzyme–intermediate complex where, analogous to the β-elimination reaction[3], the intermediate is considered to be aminocrotonate; C, β-mercaptopropionic acid; CB, *S*-carboxyethyl-L-homocysteine; and ECB, the enzyme–*S*-carboxyethyl-L-homocysteine complex. Since succinic acid is exchanged very slowly into succinylhomoserine[7], the back reaction and its rate constant are not noted. Since we are looking at the initial velocity we have ignored the reactions in which the products bind to enzyme (*i.e.* k_6 and k_{10}). If we assume that a steady state obtains during initial velocity measurements of α-ketobutyrate formation [$V_i = k_5$ (EB)] then the rate equation for the elimination reaction can be derived by the method of King and Altman[8].

$$\frac{E_0}{v_i} = \frac{1}{V}\left[\left(1 + \frac{[C]}{K_c}\right) + \left(K_{ab} + \frac{[C]}{K_x}\right)\frac{1}{[AB]}\right] \tag{4}$$

E_0 is the total enzyme concentration; v_i and V are the initial and maximal velocities of the elimination reaction; [C], the concentration of β-mercaptopropionate; [AB], the concentration of *O*-succinyl-L-homoserine; and K_{ab} is the Michaelis constant for *O*-succinyl-L-homoserine. V, K_{ab}, K_c, and K_x are defined in terms of rate constant as follows:

$$V = \frac{k_3 k_5}{k_3 + k_5}, \quad K_{ab} = \frac{k_5(k_2 + k_3)}{k_1(k_3 + k_5)}, \quad K_c = \frac{(k_8 + k_9)(k_3 + k_5)}{k_7(k_3 + k_9)},$$

$$K_x = \frac{k_1(k_3 + k_5)(k_8 + k_9)}{k_7 k_9(k_2 + k_3)}$$

Eqn 4 requires that a plot of E_0/v_i against the reciprocal of the concentration of *O*-succinyl-L-homoserine, at different fixed concentrations of β-mercaptopropionate, yields a set of straight lines with different slopes. The data illustrated in Fig. 3A are in accordance with this. Of interest is the fact that all the straight lines intersect on the abscissa. The equation also predicts that, with *O*-succinyl-L-homoserine as the independent variable, a plot of E_0/v_i against the concentration of β-mercaptopropionate will yield a set of straight lines of different slopes. This prediction is corroborated (Fig. 3B). The intercepts on the ordinate in Fig. 3A are defined as

$$\frac{E_0}{V'} = \frac{E_0}{V}\left(1 + \frac{[C]}{K_c}\right)$$

A plot of the intercepts *vs* [C] must yield a straight line. This too is borne out (Fig. 3C). From Fig. 3 the following values are obtained; K_{ab} is 0.25 mM, and K_c is 1.7 mM.

β-Mercaptopropionic acid, along with *O*-succinyl-L-homoserine, is a substrate in the γ replacement reaction yielding as product the amino acid *S*-carboxyethyl-L-homocysteine. A kinetic analysis of this reaction was performed by the indirect approach of following the more easily measurable γ elimination reaction. The data obtained are consistent with the proposed reaction scheme (Scheme 2). The kinetic analysis employed is comparable to that used in the analysis of β elimination and replacement reactions catalyzed by tryptophanase from *Escherichia coli*[3]. This type of analysis is relatively insensitive and, in the case of the γ replacement reaction under scruti-

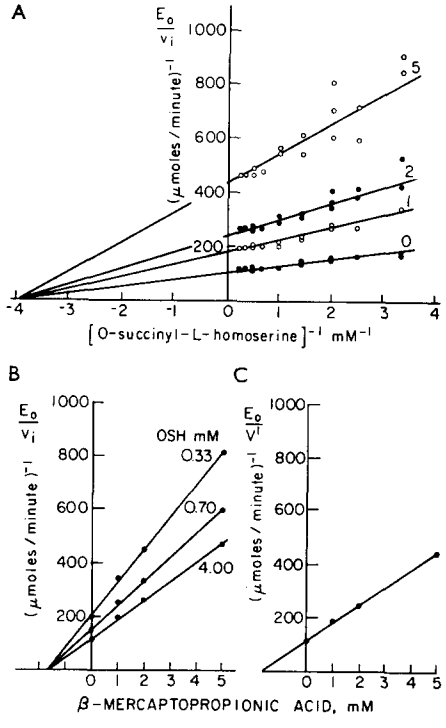


Fig. 3. α-Ketobutyrate formation from *O*-succinyl-L-homoserine catalyzed by cystathionine synthase, and its inhibition by β-mercaptopropionate. The rate of α-ketobutyrate formation from *O*-succinyl-L-homoserine, in the presence and absence of β-mercaptopropionate, was carried out, as previously described[2], by following the decrease in absorbance at 340 nm in the presence of excess NADH and lactic dehydrogenase .The reaction mixture contained 3.7 μg of cystathionine synthase (spec. act. 20) in a final volume of 1.0 ml. The temperature was 28 °C and the reaction was initiated by adding cystathionine synthase which had been prewarmed to 28 °C. Initial velocities represent α-ketobutyrate formation during the interval 60 to 90 s after the addition of the enzyme. This interval was chosen because of a lag period of up to 60 s prior to the attainment of a linear rate of reaction. The extent of substrate utilization during this lag period did not exceed 2% (in the presence or absence of β-mercaptopropionate). The lag period could not be abolished by preincubating at 37 °C or altering the order of addition of reactants. (A) Double-reciprocal plots of initial velocity with respect to concentration of *O*-succinyl-L-homoserine at fixed concentrations of β-mercaptopropionate, indicated in mM on each line. (B) Reciprocal plots of initial velocity against concentrations of β-mercaptopropionate at several fixed concentration of *O*-succinyl-L-homoserine as indicated on each line. (C) Secondary plot of E_0/V (from A) against concentration of β-mercaptopropionate.

ny, the data are consistent with several other reaction schemes. Thus, a scheme postulating a random addition of substrates with rapid equilibration of all enzyme forms can be shown to explain the data. Also, a scheme in which succinic acid is cleaved off after rather than before the binding of β-mercaptopropionate can be shown to be consistent with the observations. We have concentrated on the reaction sequence of Scheme 2 since it is the simplest scheme consistent with the data and is analogous to the postulated mechanism of β replacement and elimination[3].

Of particular interest is the observation that the K_m for *O*-succinyl-L-homoserine (0.25 mM) is unaffected by β-mercaptopropionic acid. This along with the observation that β-mercaptopropionic acid has no effect at all on cystathionine synthase-

catalyzed α and β hydrogen exchange in L-alanine[9] suggests that this compound has little effect on enzyme conformation. In previous studies it was indicated that L-cysteine increased the K_m for *O*-succinylhomoserine[2]. The existence of a lag period in the rate of α-ketobutyrate formation (legend to Fig. 3) precluded effective kinetic studies of γ replacement with L-cysteine since, after 60 s of reaction, a significant amount of substrate had been consumed by this much more rapid γ replacement reaction[2]. We suggest that the analysis leading to this earlier conclusion was not sufficiently complete and that L-cysteine may well resemble β-mercaptopropionate in having no effect on the enzyme's affinity for *O*-succinylhomoserine. Though L- and D-cysteine have been shown to inhibit total hydrogen exchange in alanine there is no effect on the differential rates of α and β exchange[9]. This suggests that cysteine acts as an inhibitor by competing for Schiff base formation and perhaps by inactivating enzyme through thiazolidine formation[10] rather than by affecting enzyme conformation and changing reactivity at the catalytic site.

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