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NEW INSIGHTS INTO THE ACTIVE CENTER OF RAT LIVER CYSTATHIONASE

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Summary

Treatment by urea of purified rat liver cystathionase (L-Cystathionine cysteine-lyase (deaminating), EC 4.4.1.1) provoked a similar alteration of two activities of the enzyme, namely cysteine desulfhydration and homoserine deamination. Since the decreases of the two activities were also comparable as a result of chymotrypsin digestion of the enzyme, these observations suggest that the two sites responsible for the one and the other activities are in close proximity.

Studies of the effect of derivatives of substrates (*S*-carboxymethylcysteine, *S*-carboxyethylcysteine, *S*-carboxymethylhomocysteine and *S*-carboxyethylhomocysteine) on both activities were performed. All of them inhibited cysteine desulfhydration and homoserine deamination; in several cases, the type of inhibition was also determined. The results are in agreement with the hypothesis that each of the two sites of the active center has, at least, three binding points which 'recognise' groupings of substrates or of inhibitors, and this led us to propose a model for the active center.

Each site has an $-NH_2$ binding point, hence the active center has two $-NH_2$ binding points; therefore, as cystathionase consists of four subunits and contains four molecules of pyridoxal phosphate, it might be of interest to determine whether the smallest active molecule is the dimer.

Introduction

It has been reported [1] and confirmed [2] that the active center of rat liver cystathionase (EC 4.4.1.1), previously referred to as L-homoserine hydro-

Abbreviations: Pyridoxal-*P*, pyridoxal phosphate; Cys(MeCOO), *S*-carboxymethylcysteine; Cys(EtCOO), *S*-carboxyethylcysteine; Hcy(MeCOO), *S*-carboxymethylhomocysteine; Hcy(EtCOO), *S*-carboxyethylhomocysteine; Hse, homoserine.

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lyase (EC 4.2.1.15) is composed of two sites, one is responsible for the desulfhydration of L-cysteine, whereas deamination of L-homoserine and desulfhydration of L-homocysteine are catalyzed by the other. In addition, some properties of the two sites were described [1]. In order to acquire further information about the sites, two kinds of experiments were set up: in the first, we determined the effect of treatment with urea as well as of limited proteolysis by chymotrypsin on the two activities, namely cysteine desulfhydration and homoserine deamination, in the second we examined the effect of derivatives of substrates (*S*-carboxymethylcysteine, *S*-carboxyethylcysteine, *S*-carboxymethylhomocysteine and *S*-carboxyethylhomocysteine) on both activities.

Here, we report the results obtained, which are consistent with our previous speculation, and we describe additional properties of the two sites. Thence, we suggest a diagram for the active center according to which each site has at least three binding points which 'recognise' groupings of the respective substrates or inhibitors.

A preliminary account of these results has already appeared [3].

Materials and Methods

Purification of cystathionase

The pattern of purification of cystathionase from Wistar rat livers was similar to that described [4,5]. However we did not neutralize the preparation at the step of $(NH_4)_2SO_4$ fractionation as we observed that under those conditions, after a run on a Sephadex G-25 column, only peak B was active and contained the majority of the original activity. Furthermore, when the active fractions (peak B) were pooled and dialysed overnight in the cold against several changes of 0.02 M phosphate buffer (pH 5.6), a precipitate was formed in the bag, which, collected by centrifugation, could be redissolved in 0.06 M phosphate buffer (pH 7.8) containing $2 \cdot 10^{-4}$ M pyridoxal-*P* and 10^{-4} M EDTA, giving a satisfactory yield of purification. Although thorough study was not performed at this stage, it could be suggested that precipitation of the enzyme occurs as, under those conditions, the enzyme is at a pH value very near to its isoelectric point; rat liver cystathionase is indeed an acid protein [2,6]. Anyhow the adoption of these modifications clearly decreased the time consumed for the enzyme purification and also gave a purified preparation whose specific activity was similar to that previously obtained.

The enzymatic activities were determined as reported [1,4,5], with two slight modifications: all experiments were carried out in phosphate buffer (pH 7.8) and in every case pyridoxal-*P* ($2 \cdot 10^{-4}$ M) and dithiothreitol (10^{-3} M) were included in the assay. Whenever the effect of the derivatives of substrates on the activities was investigated, the derivative, in solution suitably adjusted to pH 7.8, was added to the enzyme prior to the addition of the substrate.

The initial rates are expressed as μmol of H_2S or α-ketobutyric acid produced $\cdot h^{-1} \cdot mg^{-1}$ protein, L-cysteine or L-homoserine, respectively, being the substrate.

Treatment with urea and with chymotrypsin

As it has been observed that pyridoxal-*P* partially protected cystathionase

against urea inactivation [7] and also against chymotrypsin digestion [8] and as pyridoxal-*P* is routinely included in the buffers used in the purification, in order to increase the stability of the enzyme [4], a sample of the purified enzyme, containing 9 mg of protein/ml was dialysed overnight in the cold against 0.02 M phosphate buffer (pH 5.6) containing 10^{-4} M dithiothreitol but devoid of pyridoxal-*P*, and the precipitate, collected by centrifugation, was redissolved in 0.06 M phosphate buffer (pH 7.8) containing 10^{-4} M dithiothreitol but devoid of pyridoxal-*P*. The activity of this sample, measured in absence of pyridoxal-*P* in the incubation mixture (L-homoserine or L-cysteine being used as substrate) was less than 10% of the activity measured in the presence of added coenzyme, which indicates that the preparation is mainly on the form of apocystathionase. This sample was used for the determination of the effect of urea and of chymotrypsin on the two activities.

On the one hand, aliquots of this preparation were incubated with or without urea (2.5 M) for 45 min at 37° C. Both activities of the control and of the urea treated enzyme were measured after suitable dilution. On the other hand, aliquots were incubated with or without chymotrypsin (10 μg/ml of the incubated mixture) in phosphate buffer (pH 7.8) for 30 min at 37° C, the two activities were measured on the control and on the chymotrypsin-treated enzyme suitably diluted. Under both conditions we checked that the final concentration of urea (or of chymotrypsin) in the assay was without significant effect when added to the untreated enzyme.

Synthesis of the derivatives of the substrates

Chemical synthesis of the derivatives of cysteine and of homocysteine were carried out by one of us (P.M.) according to methods which will be described elsewhere.

Results

(1) Effect of treatment with urea and with chymotrypsin

In Table I (expressed as percentage of inhibition) are the activities of cysteine desulfhydration and of homoserine deamination (in each case the mean of five experiments) of the treated sample, incubated with urea and with chymotrypsin. On the one hand, as the enzyme is mainly in the form of apocystathionase, the small decrease in activity of the controls resulting from incubation is in agreement with previous observations [7,8]. On the other hand, it is obvious that, whatever the inactivating agent employed, both activities decreased similarly. Indeed the percentages of inhibition were 80% for cysteine desulfhydration and 70% for homoserine deamination when treatment with urea was performed, and respectively 60% and 50% when chymotrypsin was employed. It has been already reported [9] that treatment of cystathionase with dodecyl sulfate provoked a similar decrease in the two activities. Therefore, taken as a whole, the results of these experiments suggest that the two sites are in close proximity since they are influenced by the same factors.

(2) Effect of substrate derivatives

First we ascertained that none of the derivatives of substrates was a sub-

TABLE I

EFFECT OF UREA AND OF CHYMOTRYPSIN ON THE TWO ACTIVITIES OF A SAMPLE OF PURIFIED CYSTATHIONASE DEPRIVED OF PYRIDOXAL-*P* PRIOR TO THE TREATMENTS

C: control (cystathionase alone); T: treated (cystathionase + urea (or + chymotrypsin)). The incubations were performed as indicated in the text. The determination of activities was made in presence of pyridoxal-*P* in the assay. The values are the percentages of inhibition with regards to the activity of the untreated sample.

	Urea (2.5 M)		Chymotrypsin (10 μg/ml)	
	C	T	C	T
Cys desulfhydration	7.5	80	10	60
Hse deamination	2.5	70	5	50

strate for cystathionase. However, we observed that all of them inhibited the desulfhydration of cysteine and deamination of homoserine: a general summing up of the results obtained is given in Table II. Furthermore, in several cases, the type of inhibition produced by the derivative on both activities was determined and the results are shown in Table III.

S-Carboxyethylhomocysteine (Hcy(EtCOO))

Hcy(EtCOO) was not a substrate for cystathionase although its structure is very similar to that of cystathionine (the only change being that the $-NH_2$ group of the "cysteine" part of the molecule is missing). As cystathionine is a very good substrate [10] we can conclude that the presence of this $-NH_2$ group is a prerequisite for the deamination of the "homoserine" part of the molecule

TABLE II

PERCENTAGES OF INHIBITION BY DERIVATIVES OF THE SUBSTRATES ON THE ACTIVITIES OF HOMOSERINE DEAMINATION AND OF CYSTEINE DESULFHYDRATION OF PURIFIED RAT LIVER CYSTATHIONASE

Cys(MeCOO) = *S*-carboxymethylcysteine; Cys(EtCOO) = *S*-carboxyethylcysteine; Hcy(MeCOO) = *S*-carboxymethylhomocysteine; Hcy(EtCOO) = *S*-carboxyethylhomocysteine. The values are the percentages of inhibition observed when the derivatives, at the indicated concentration, were included in the assays. The concentration of the substrate (homoserine or cysteine) in the assays was $5 \cdot 10^{-2}$ M. The determination of activity was performed, as stated in the text, in presence of pyridoxal-*P*.

Derivatives	Concn (M)	Hse deamination	Cys desulfhydration
Cys(MeCOO)	10^{-3}	25	20
	10^{-2}	65	45
	$5 \cdot 10^{-2}$	86	77
Cys (EtCOO)	10^{-3}	25	20
	10^{-2}	65	35
	$5 \cdot 10^{-2}$	80	40
Hcy(MeCOO)	10^{-3}	25	37
	10^{-2}	50	60
Hcy(EtCOO)	10^{-3}	10	0
	10^{-2}	47	27
	$5 \cdot 10^{-2}$	74	62

TABLE III

TYPE OF INHIBITION PRODUCED BY DERIVATIVES OF THE SUBSTRATES ON HOMOSERINE DEAMINATION AND CYSTEINE DESULFHYDRATION

C = competitive inhibition; N.C. = non competitive inhibition. V is expressed as μM of product $\cdot h^{-1} \cdot mg^{-1}$ protein; the values of K_i were determined in the presence of the substrate for the other site (respectively Hse or Cys). The abbreviations are the same than those of Table II; Met = methionine.

Derivatives	Concn (M)	Hse deamination				Cys desulfhydration			
		Type of inhibition	K_m (10^{-2} M)	V	K_i (10^{-3} M)	Type of inhibition	K_m (10^{-2} M)	V	K_i (10^{-3} M)
Hcy(EtCOO)	10^{-3}	C	1.1	740	3	No inhibition			
	10^{-2}	C	1.1	740	3	C	1.0	55	3.5
	$2 \cdot 10^{-2}$					C	1.0	55	3.5
Cys(EtCOO)	10^{-3}	Mixed	1.5	520		N.C.	2.5	50	4
	10^{-2}	Mixed	1.5	520		Complex	2.5	50	
Cys (MeCOO)	10^{-3}	N.C.	1.5	550	3.7	N.C.	1.9	38	3.3
	$5 \cdot 10^{-3}$	Mixed	2.3	660					
	10^{-2}	Mixed	2.3	660		Complex	1.4	50	
	$2 \cdot 10^{-2}$					Complex	1.4	50	
Hcy(MeCOO)	10^{-3}	N.C.	1.3	450	3.3	N.C.	1.3	50	1.4
	10^{-2}	Mixed	1.3	450		Mixed	1.3	50	
Cys(MeCOO) + Met (10^{-1} M)	10^{-3}					No inhibition			
	10^{-2}					C	2.1	44	6.7
Hcy(MeCOO) + Met (10^{-1} M)	10^{-3}					No inhibition			
	10^{-2}					C	2.5	60	6.1

although it is itself not attacked as deamination of cystathionine is associated with a release of cysteine [11].

However, we observed that Hcy(EtCOO) was a competitive inhibitor of homoserine deamination and, when its concentration was high enough, also a competitive inhibitor of cysteine desulfhydration (Table III).

Assuming that a molecule of cystathionine fits on the two sites of the active center, that is to say the "homoserine site" (which we call C_4, and was previously referred to as the L site [1]) and the "cysteine site" (which we call C_3, and was previously referred to as the DL site [1]), we can also assume that a similar situation arises for Hcy(EtCOO). Hence, the competitive inhibition of homoserine deamination and of cysteine desulfhydration might result from the binding of the "homoserine" part of Hcy(EtCOO) on the site C_4 and of the "cysteine" part of Hcy(EtCOO) on the site C_3, respectively. As a matter of consequence we can infer that L-cystathionine might be a competitive inhibitor of both deamination of homoserine and desulfhydration of cysteine.

The fact that a high concentration of Hcy(EtCOO) is necessary for inhibiting desulfhydration of cysteine might reflect the non-complete adequacy of this derivative for site C_3 whereas the competitive inhibition of homoserine deamination, whatever the concentration of Hcy(EtCOO), might result from the occupation of site C_4 by the "homoserine" part of the molecule. It is

noticeable that Hcy(EtCOO) inhibits more efficiently the deamination of homoserine than the desulfhydration of cysteine (Table II).

S-Carboxymethylcysteine (Cys(MeCOO))

Hcy(MeCOO) was an inhibitor of both reactions and was slightly more effective on the desulfhydration of cysteine than on the deamination of homoserine (Table II). At 10^{-3} M, Hcy(MeCOO) was a non competitive inhibitor of homoserine deamination, and at higher concentrations the inhibition was of a mixed type (Table III and Fig. 1).

Similar observations were made as regards the desulfhydration of cysteine. Thus, we can envisage that the binding of the carboxymethyl part of Hcy(MeCOO) on site C_3 provoked the non-competitive inhibition of homoserine deamination and, conversely, that binding of the "homoserine" part of Hcy(MeCOO) on site C_4 was responsible for the non-competitive inhibition of desulfhydration of cysteine. On the other hand, when the concentration of Hcy(MeCOO) was 10^{-2} M, the mixed type inhibition observed for both reactions results from the superposition of two kinds of inhibition: one non-competitive and the other competitive. The possibilities for explaining these observations will be examined in the discussion. In this connection it is worthy of note that the affinities of sites C_3 and C_4 for Hcy(EtCOO) and Hcy(MeCOO), respectively measured in the presence of the substrate for the other site, are similar (Table III) and are approximately the same as the affinity to the active center for cystathionine [12,13] which means that they are about ten-fold the affinity of the active center for L-homoserine [12] and for L-cysteine [14] measured when present alone. As the effect of Hcy(MeCOO) on the two activities of cystathionase was not the same as the effect of Hcy(EtCOO), these observations seriously suggest that the mechanisms of action of both derivatives on the enzyme are different.

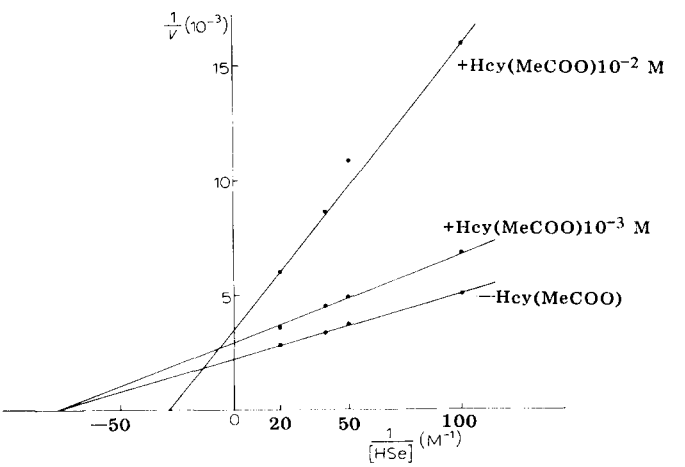


Fig. 1. Effect of Hcy(MeCOO) on deamination of homoserine. Hcy(MeCOO) = *S*-carboxymethylhomocysteine. A Lineweaver and Burk plot is given. The reaction was carried out at 37° C for 20 min. The incubated mixture contained 0.2 M phosphate buffer (pH 7.8), 10^{-3} M dithiothreitol and $2 \cdot 10^{-4}$ M pyridoxal-*P*. The initial rates are expressed as $\mu mol \cdot h^{-1} \cdot mg^{-1}$ of enzyme.

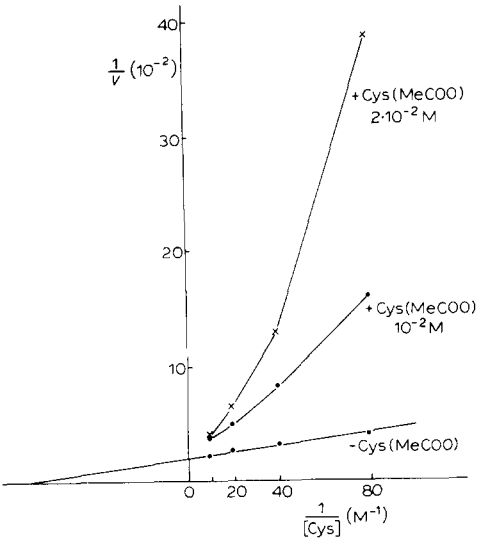


Fig. 2. Effect of 10^{-2} M and $2 \cdot 10^{-2}$ M Cys(MeCOO) on desulfhydration of cysteine. Cys(MeCOO) = *S*-carboxymethylcysteine. The conditions are similar to those described in the legend to Fig. 1.

S-Carboxymethylcysteine (Cys(MeCOO)

Cys(MeCOO) inhibited both activities (Table II), the percentages of inhibition being approximately the same for both reactions. Table III shows that homoserine deamination was non-competitively inhibited when the concentration of Cys(MeCOO) was 10^{-3} M, and that the inhibition was of a mixed type when the concentration of Cys(MeCOO) was 10^{-2} M. For cysteine desulfhydration, the inhibition was non-competitive with 10^{-3} M Cys(MeCOO) (Table III) but far more "complex" at 10^{-2} M (Fig. 2).

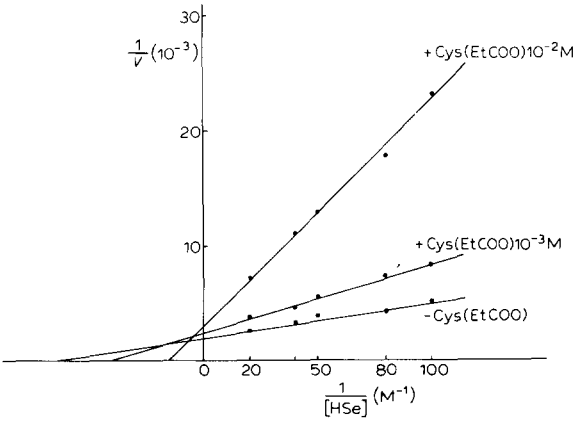


Fig. 3. Effect of 10^{-3} M and 10^{-2} M Cys(EtCOO) on deamination of homoserine. Cys(EtCOO) = *S*-carboxyethylcysteine. The conditions are similar to those described in the legend to Fig. 1.

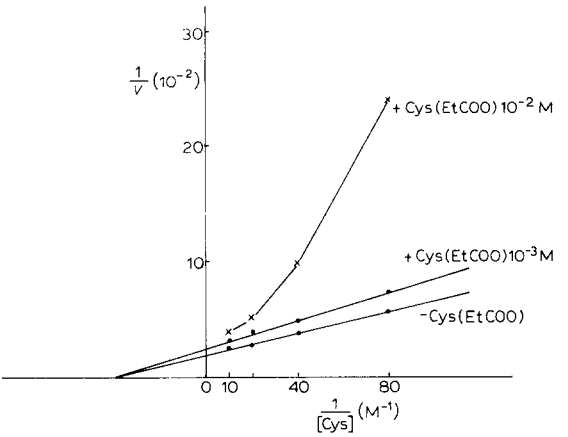


Fig. 4. Effect of 10^{-3} M and 10^{-2} M Cys(EtCOO) on desulfhydration of cysteine. Cys(EtCOO) = *S*-carboxyethylcysteine. The conditions are similar to those described in the legend to Fig. 1.

S-carboxyethylcysteine (Cys(EtCOO))

Cys(EtCOO) inhibited both activities but this derivative acted more on homoserine deamination than on cysteine desulfhydration, mainly at concentrations of 10^{-2} M and $5 \cdot 10^{-2}$ M (Table II; Fig. 3).

The inhibition was of mixed type for homoserine deamination (Table III) and was non-competitive for cysteine desulfhydration with 10^{-3} M Cys(EtCOO) while a "complex" inhibition was observed at 10^{-2} M (Fig. 4).

Effect of Cys(MeCOO) and of Hcy(MeCOO) in presence of methionine

As it has been reported [1] that 10^{-1} M methionine competitively inhibited the deamination of homoserine, whereas it did not alter cysteine desulfhydration, some experiments were carried out in presence of methionine.

We observed that, under these conditions, Cys(MeCOO) at 10^{-3} M did not

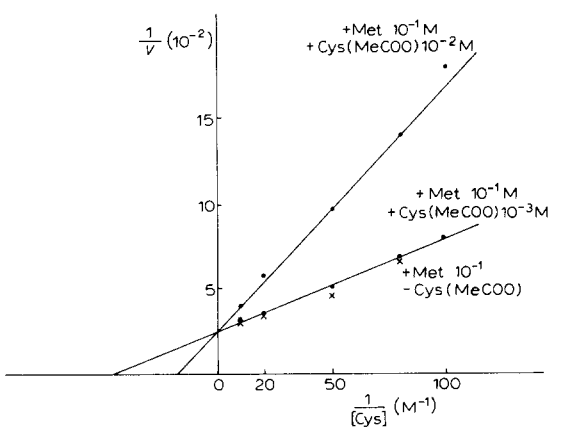


Fig. 5. Effect of 10^{-3} M and 10^{-2} M Cys(MeCOO) on desulfhydration of cysteine in the presence of 10^{-1} M methionine. Cys(MeCOO) = *S*-carboxymethylcysteine. The conditions are similar to those described in the legend to Fig. 1. X———X, control; ●———●, 10^{-3} M and 10^{-2} M Cys (MeCOO).

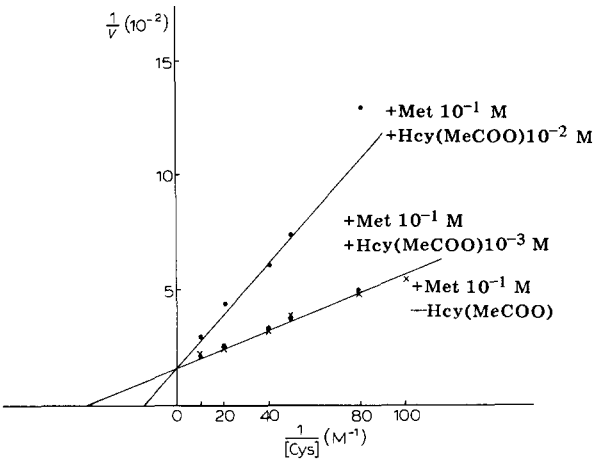


Fig. 6. Effect of 10^{-3} M and 10^{-2} M Hcy(MeCOO) on desulfhydration of cysteine in presence of 10^{-1} M methionine. Hcy(MeCOO) = *S*-carboxymethylhomocysteine. The conditions are similar to those described in the legend to Fig. 1. X———X, control; •———•, 10^{-3} M and 10^{-2} M Hcy(MeCOO).

inhibit cysteine desulfhydration, and that at 10^{-2} M the inhibition of this reaction was competitive (Table III; Fig. 5).

These results could be interpreted as follows: assuming that methionine is bound to site C_4 and thence prevents the binding of either "carboxymethyl" part of "cysteine" part of Cys(MeCOO) to this site, the competitive inhibition or cysteine desulfhydration should result from the binding of the "carboxymethyl" part or the "cysteine" part of Cys(MeCOO) to site C_3. These possibilities will be examined in the discussion.

Similar results were obtained when methionine was used at the same time than Hcy(MeCOO), that is to say that, under these conditions, the inhibition of cysteine desulfhydration was competitive (Fig. 6).

The interpretation which may be retained is the following: assuming that methionine is bound to site C_4 and prevents the binding of the "homocysteine" part of Hcy(MeCOO) to site C_4, we have to envisage that a part of the molecule of Hcy(MeCOO) is bound to site C_3 in such a way that a competitive inhibition between this part and cysteine occurs. Owing to the fact that site C_3 does not bind substances having more than three atoms of carbon in their structure [1] the "homocysteine" part of Hcy(MeCOO) is excluded and the only possibility left to consider is the binding of the "carboxymethyl" part of Hcy(MeCOO) to site C_3. This will be examined in the discussion.

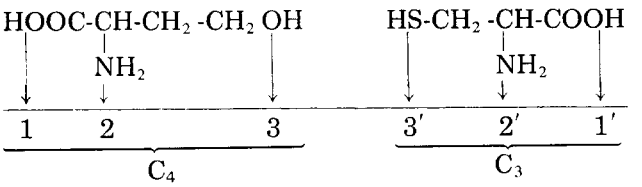
Discussion and Conclusions

These results give new evidence that the active center of rat liver cystathionase constitutes a site (we call it C_3) for the desulfhydration of cysteine and another site (we call it C_4) for the deamination of homoserine. Furthermore, the findings that urea and chymotrypsin, respectively, modified both reactions in a similar way, as well as previous observations [9] according to which treatment of cystathionase with sodium dodecyl sulfate provoked a similar

decrease of both activities, suggest that the two sites are in close proximity since they are influenced by the same factors. This suggestion is in keeping with the synthesis, by cystathionase, of cystathionine from homoserine and cysteine [15,16] and also, as recently reported [17], from homocysteine and cysteine.

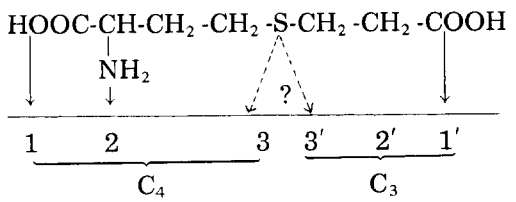
The derivatives of the substrates (*S*-carboxymethylcysteine, *S*-carboxyethylcysteine, *S*-carboxymethylhomocysteine and *S*-carboxyethylhomocysteine) are inhibitors of both reactions. At high concentrations (10^{-2} M and $5 \cdot 10^{-2}$ M), the two carboxymethyl derivatives of the substrates are more potent inhibitors of the cysteine desulfhydration than the two carboxyethyl derivatives. As regards homoserine deamination, the two derivatives of cysteine have a similar efficiency and are a little more effective than the derivatives of homocysteine.

Taken as a whole, these findings led us to propose a diagram for the active center which is illustrated below, homoserine and cysteine being respectively taken as representative substrates for C_4 and C_3.



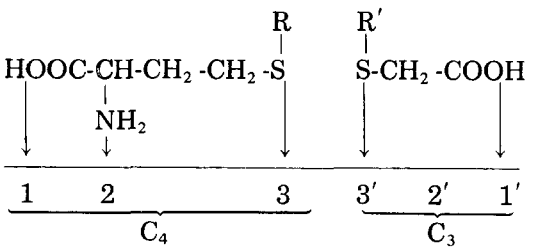
In other words, the site C_4 has three "binding points", one for the -COOH group, one for the $-NH_2$ group and one for the oxygen atom. Similarly, the site C_3 has also three "binding points", one for the -COOH group, one for the $-NH_2$ group and one for the sulfur atom. The reasons underlying this assumption are that all the substrates of cystathionase are "simple" or "double" aminoacids [1]. At the moment we have no information about the nature of the binding points for -COOH groups and for sulfur and oxygen atoms, but it is very likely that the binding points for $-NH_2$ groups are the molecules of pyridoxal-*P* of the enzyme. It is worthy to note that homoserine is a better substrate [10] than homocysteine although their K_m values for cystathionase are approximately the same [4,12]; thence, on site C_4 the degradation of a substrate having an atom of oxygen (homoserine) is favoured when compared to that of a substrate possessing an atom of sulfur (homocysteine). However, on site C_3, L-cysteine is efficiently degraded whereas L-serine is a very poor substrate [10,18] although it inhibits desulhydration of cysteine [1], which indicates that position 3' of site C_3 preferentially recognizes or preferentially releases a sulfur atom.

The results obtained with *S*-carboxyethylhomocysteine can be visualized as follows:



Indeed we do not know whether the sulfur atom of this derivative is bound to point 3 of site C_4 or to point 3′ of site C_3. A similar situation also arises for cystathionine which is a very good substrate for cystathionase. However, as far as *S*-carboxyethylhomocysteine is concerned, the competitive inhibition of homoserine deamination might result from the binding of this molecule to point 1 and 2 (and perhaps 3) of site C_4 while the competitive inhibition of cysteine desulfhydration might reflect the binding of groups of *S*-carboxyethylhomocysteine to position 1′ (and may be also to point 3′) of site C_3. An hypothesis worthwhile exploring can be suggested: assuming that the sulfur atom of this derivative is bound to position 3′ of site C_3 and thence that two binding points of each site are occupied by groups of a molecule of this derivative, it follows that competitive inhibition may occur when only two of the three binding points of a site are occupied by groups of one molecule of an inhibitor. Whatever the situation might be, it is obvious that a molecule of *S*-carboxyethylhomocysteine fits both sites of the active center very well, presumably because its ressemblance to cystathionine favours the combining.

The interpretation of the results obtained with *S*-carboxymethylhomocysteine is a little more complicated: indeed as the inhibition observed at 10^{-3} M is non-competitive for both reactions and becomes of mixed type at 10^{-2} M (Table III) it is not possible to retain an hypothesis implicating the involvement of only one molecule of the derivative. Therefore, we can put forward an hypothesis implicating the involvement of two molecules of the derivative, and illustrated as follows:



in which R = $-CH_2-COOH$ and R′ = $-CH_2-CH_2-CH(NH_2)COOH$.

This hypothesis is in keeping with the results obtained with 10^{-2} M *S*-carboxymethylhomocysteine: the inhibition of mixed type homoserine deamination results from the competitive inhibition produced by the binding of the "homoserine" part of one molecule of the derivative to site C_4, and, on the other hand, the non-competitive inhibition of homoserine deamination is due to the binding of the *S*-carboxymethyl part of another molecule of the derivative to site C_3. As the K_i value (dissociation constant of the complex cystathionase-*S*-carboxymethylhomocysteine) is approximately (Table III) one tenth the K_m value of the enzyme for cysteine, it results that the affinity of the active center for *S*-carboxymethylhomocysteine is approximately ten-fold the affinity of the active center for cysteine. We can therefore suggest that when the concentration of the derivative is 10^{-2} M, the binding of the *S*-carboxymethyl part of one molecule of the derivative on site C_3 might competitively inhibit the desulfhydration of cysteine. This suggestion is in agreement with the results obtained in presence of *S*-carboxymethylhomocysteine and methionine.

It seems that this hypothesis is the most likely and thence worthy to explore. Also we can stress that the binding of one molecule of *S*-carboxymethylhomocysteine to the two sites of the active center would presumably create "distorsion" of the active center while the involvement of two molecules would not. Although other more complicated possibilities cannot be excluded, the results obtained with *S*-carboxymethylcysteine and *S*-carboxyethylcysteine could also be interpreted in a similar way, all the more as the "cysteine" part of these derivatives can bind site C_4 as well as site C_3.

In conclusion, these observations suggest that, whatever it might be, a similar mechanism involving two molecules of the inhibitor may be retained for interpreting the results obtained with *S*-carboxymethylcysteine, *S*-carboxyethylcysteine and *S*-carboxymethylhomocysteine; whereas, in the case of *S*-carboxyethylhomocysteine, only one molecule is implicated as this molecule appears as well adapted to the active center.

As far as the active center itself is concerned, it is noticeable that some kind of "flexibility" (or possibilities of some "distorsion" of the two sites) exists since lanthionine [10], djenkolic acid [10,19], cystine [20] and diaminopropionic acid [18] are substrates of cystathionase.

The diagram we suggest for the active center of rat liver cystathionase is consistent with a large number of the observations already made about this enzyme and provides the basis for further investigations.

For instance, according to this diagram the active center has two molecules of pyridoxal-*P*, since one of them is bound to each site. Previously Matsuo and Greenberg [12] determined that one molecule of cystathionase binds four molecules of pyridoxal-*P*. On the other hand, it has been observed in our laboratory [9] that rat liver cystathionase consists of four subunits, whereas Churchich and Dupourque [21] concluded that the enzyme consists of eight subunits. In spite of this discrepancy it might be of interest to investigate whether the smallest active form of the enzyme is a dimer composed of two subunits each of them bearing one site (C_3 or C_4). Indeed, we already know that the subunit we obtained, whose molecular weight is around 44 000, is devoid of enzymic activity towards L-homoserine and L-cysteine.

Whatever the situation might be, the physiological implications of our observations may be of importance: indeed, Hcy(MeCOO) and Cys(EtCOO) are present in fairly large amounts in the urine of cystathioninuric patients [22,23], Cys(MeCOO) was described as occuring in the urine of patients suffering from diabetes and hypertension [24], Hcy(MeCOO) [25] and Hcy(EtCOO) [26] were found in the urine of homocystinuric patients.

As far as cystathioninuria is concerned, assuming that the high amounts of Hcy(MeCOO) and Cys(EtCOO) present in the urine of patients reflect high concentrations of these substances in their tissues, it might be of interest to investigate whether they contribute to the low activity of cystathionase [27] observed in the liver of these patients. On the other hand, in the liver of homocystinuric patients, the activity of cystathionine synthase is either very low or even absent [28] whereas the activity of cystathionase is not impaired [29]. Thence, a biosynthesis of cystathionine from homoserine and cysteine due to cystathionase acting in the "reverse" direction [10,16] has been suggested as a way to correct cystathionine deficiency [15].

Experimental results strongly support this suggestion since intraperitoneal injections of homoserine and cysteine in the mouse resulted in a marked increase in brain and liver cystathionine [30,31], and oral administration of these substances to homocystinurics resulted in urinary excretion of cystathionine [15,32]. However assuming that the urinary excretion of Hcy(MeCOO) and Hcy(EtCOO) reflects the presence of these substances in the tissues of homocystinuric patients, one wonders whether they exist at a concentration high enough, when compared to the concentration of the substrates of the enzyme, to effectively inhibit, in vivo, the activity of cystathionase.

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