FUNCTIONAL ARGININE IN THE ACTIVE CENTER OF RAT LIVER CYSTATHIONASE

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1. Introduction

A model for the active center of rat liver cystathionase (EC 4.4.1.1.) has been proposed according to which each of the two sites has at least three binding points for grouping of the substrates L-homoserine and L-cysteine, respectively [1]. The residues of the enzyme necessary for the binding of these groupings are still unknown. There is increasing evidence that in a wide variety of enzymes arginyl residues are functional for the binding of substrates or coenzymes, especially in enzymes acting on carboxyl- or phosphoryl-containing substrates [2–10]. Since butanedione under the conditions described by Riordan [11] provides a remarkable tool for the study of arginine, we investigated the effect of butanedione on cystathionase.

2. Materials and methods

Rat liver cystathionase was purified as described previously [12,13]. The enzyme, obtained as holoenzyme, was homogeneous, as judged by electrophoresis on cellogel strips, and was lyophilized to be conveniently stored. Measurement of activity was carried out as already described [13]; however, the buffer used was either 0.2 M phosphate, pH 7.8, or 0.07 M veronal, pH 8.6, or 0.07 M borate, pH 8.6, as the 2,3-butanedione monoxime solution (Eastman Kodak) was prepared in these different buffers. In every case the same buffer was used for the dissolution of the enzyme, butanedione and the measurement of activity. The temperature of incubation was either 4°C or 20°C. After suitable dilution of the incubated mixture, the concentration of butanedione in the assay was checked to be without effect on the activity, when added to a non-incubated sample of enzyme. When the effect of substrates or of other substances was investigated, each of these products was added to the enzyme prior to the addition of butanedione and, in these cases, the temperature of incubation was 4°C.

3. Results

3.1. *Inactivation of cystathionase by butanedione*

We observed that whatever the buffer used and whatever the temperature of incubation the activities of the butanedione-treated enzyme were decreased when compared to those of sample of enzyme incubated in the same buffer without butanedione. For instance after 15 min incubation at 20°C with 6.7×10^{-1} M butanedione in veronal buffer the activity of homoserine deamination was roughly 10% of the initial activity of the enzyme whereas the activity of cysteine desulfhydration was nil.

Figure 1 shows the time course of the inactivation of homoserine deamination and of cysteine desulfhydration by butanedione (3.3×10^{-1} M) in borate buffer at 20°C.

Figure 2 shows the inactivation of both activities when the incubation was performed for 20 min at 4°C in phosphate or in borate buffers with various concentrations of butanedione.

It is obvious that whatever the conditions the inactivation of cysteine desulfhydration is higher than the inactivation of homoserine deamination

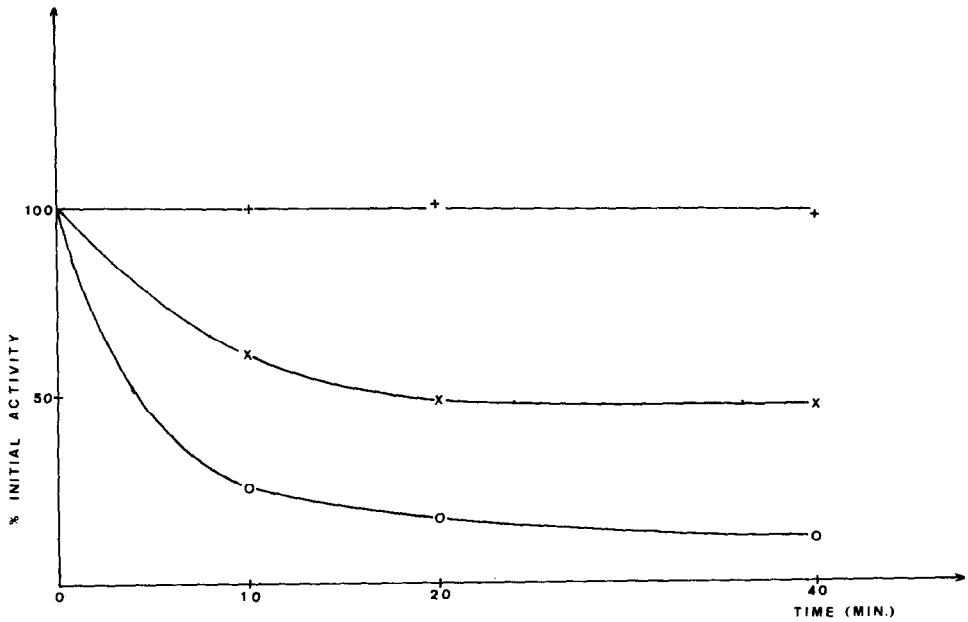

Fig.1. Time course of inactivation of cystathionase by butanedione. Lyophilized enzyme was dissolved (2 mg/ml) in 0.07 M borate buffer, pH 8.6 and incubated at 20°C with 3.3×10^{-1} M butanedione in the same buffer. (+–+) Control (for both activities). (X–X) Homoserine deamination. (0–0) Cysteine desulfhydration.

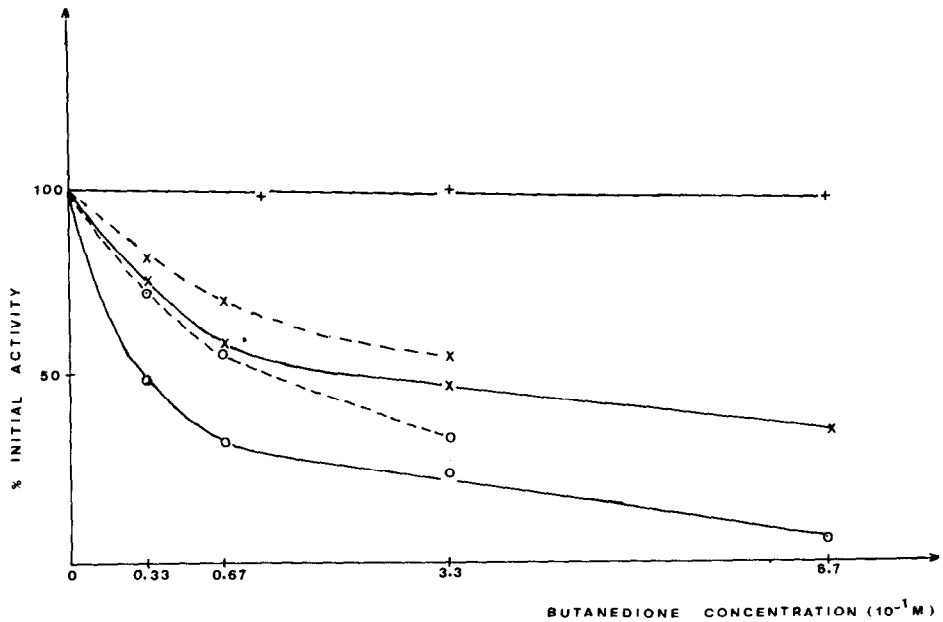

Fig.2. Effect of the concentration of butanedione on the inactivation of cystathionase. Lyophilized enzyme was dissolved (2 mg/ml) in 0.07 M borate buffer, pH 8.6 or in 0.2 M phosphate buffer, pH 7.8 and incubated for 20 min at 4°C with various concentrations of butanedione in respectively borate buffer or phosphate buffer. (——) Incubation in borate buffer. (– – – –) Incubation in phosphate buffer. In phosphate buffer a precipitation occurs with the highest concentration of butanedione and thence the measures of activities were not carried out. Other legends as in fig.1.

and that both inactivations are somewhat greater in borate buffer than in phosphate buffer.

As already observed for other enzymes [5] the inactivation of cystathionase due to butanedione was not reversed when the enzyme incubated with 3.3×10^{-1} M butanedione in borate buffer for 20 min at 4°C was run in the cold on a 1 × 15 cm column of Sephadex G-25 equilibrated with the borate buffer, the elution being performed with the borate buffer. Indeed the activities of the pooled protein containing fractions measured immediately after the run or after 30 min at 20°C in presence of pyridoxal phosphate (10^{-4} M) were the same in both cases and were approximately the same as those charged on the column.

3.2. *Protection against inactivation by butanedione by subtrates and by competitive inhibitors of cystathionase*

As butanedione inhibits both activities this suggests that it reacts with the one and the other sites. Therefore experiments in which L-homoserine, substrate for site C_4, was added at the final concentration 10^{-2} M to the enzyme prior to the addition of 6.7×10^{-2} M butanedione were performed at 4°C. Similarly experiments with the substrate of site C_3, L-cysteine (10^{-1} M) as potential protector, were performed. The effect of pyridoxal phosphate (10^{-4} M) was also examined. Finally as *S*-carboxyethylhomocysteine is a competitive inhibitor of cystathionase [1,14] as well for deamination of homoserine as for desulfhydration of cysteine [1] we determined the effect of this substance added at final concentration 5×10^{-3} M prior to butanedione.

The results are recorded in table 1.

The lack of effect of pyridoxal phosphate was not unexpected as the enzyme was under the form of holoenzyme. L-Homoserine afforded a significant protection for site C_4 whereas being without noticeable effect for site C_3. L-Cysteine produced a partial protection for site C_3, on which its desulfhydration occurs, and at the same time an inhibition of homoserine deamination. This last observation is in keeping with the already reported [15] inhibition of homoserine deamination by cysteine, resulting very likely from the removal of pyridoxal phosphate from site C_4. *S*-Carboxyethylhomocysteine protected both sites against inactivation by butanedione.

Table 1
Protection by substrates and by a competitive inhibitor of cystathionase against inactivation by butanedione

	Homoserine deamination	Cysteine desulfhydration
E	100	100
E + PLP	100	100
E + BD	65	47
E + PLP + BD	64	50
E + HS + BD	94	54
E + CY + BD	27	70
E + *S*-CH + BD	86	81

Abbreviations: E, cystathionase; BD, butanedione; PLP, pyridoxal phosphate; HS, L-homoserine; CY, L-cysteine; *S*-CH, *S*-carboxyethylhomocysteine

All experiments were carried out in 0.07 M borate buffer, pH 8.6. Results (means of three experiments) are expressed as percentage of initial activity

Conditions are described in the text

These results suggest that butanedione reacts with sites of recognition of grouping present in both substrates. In this respect the sites of recognition of the α-carboxyl groups of both substrates are the best candidates. On the other hand as butanedione specifically reacts, under these conditions, with arginyl residues, we tentatively conclude that the α-carboxyl groups of L-homoserine and of L-cysteine are bound to the enzyme through arginyl residues. To strenghten this assumption we used cysteamine (β-mercaptoethylamine). We observed indeed [16] that cysteamine competitively inhibits desulfhydration of cysteine. We previously suggested that competitive inhibition could result from the blocking of two binding points [1]. It is clear that cysteamine could be bound to site C_3 through the recognition sites of the sulfur atom and of the $-NH_2$ grouping but leaves free the recognition site for the –COOH grouping. We observed (table 2) that, even in presence of 5×10^{-3} M cysteamine, butanedione (6.7×10^{-2} M) in borate buffer inhibits cysteine desulfhydration.

Of particular interest is the observation that the activities of cysteine desulfhydration (expressed in percentages of initial activity) are very similar when respectively compared to the activity of the sample of enzyme on the one hand and to the

Table 2
Effect of cysteamine on the inactivation of cysteine desulfhydration by butanedione

	Activity (μmol H_2S/h/ml)	A	B
E	31	100	
E + β-ME	17.7	57	100
E + BD	13.6	44	
E + β-ME + BD	5.5	18	45

A, % initial activity
B, % activity recovered when compared to that observed in presence of β-ME

β-ME, β-mercaptoethylamine (cysteamine). Other legends as in table 1

All experiments were carried out in 0.07 M borate buffer, pH 8.6. The results are the means of two experiments

activity measured in presence of cysteamine on the other hand.

4. Discussion

Braunstein suggested [17] that, for aspartate aminotransferase, lysine or arginine residues bind the phosphate group of the coenzyme, pyridoxal phosphate and the ω-carboxyl groups of the substrates. Evidence was obtained [6,7] that the ω-carboxyl groups of the substrates are bound to the enzyme through arginyl residues. Kazarinoff and Snell [3] showed that, for D-serine dehydratase, an arginine residue is essential for the binding of the phosphate group of pyridoxal phosphate. At the moment we cannot decide whether or not, for rat liver cystathionase, arginine residues are necessary for the binding of the phosphate group of pyridoxal phosphate, as we only used the holoenzyme. However, all our observations indicate that there are functional arginyl residues in the active center of the enzyme and that the α-carboxyl groups of the substrates are bound to the enzyme through arginyl residues. This is in keeping with a recent report [18]. Therefore we conclude that the binding point 1′ of site C_3 and the binding point 1 of site C_4 of cystathionase [1] are arginyl residues.

Acknowledgements

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