NUMBER OF ARGININE RESIDUES IN THE SUBSTRATE BINDING SITES OF RAT LIVER CYSTATHIONASE

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1. Introduction

It has been reported that rat liver cystathionase (EC 4.4.1.1) is inhibited by butanedione and suggested that the α-carboxyl groups of the substrates (L-homoserine and L-cysteine, respectively) are bound to the protein through arginine residues [1]. To support the concept that specific residues are critical or even essential to enzyme activities, we carried out experiments using phenylglyoxal, another fairly specific reagent, for the chemical modification of arginine residues in proteins [2]. Two kinds of experiment were carried out:

(i) [^{14}C]Phenylglyoxal (CEA, Saclay) was used to check whether radioactivity was incorporated into cystathionase.
(ii) Phenylglyoxal monohydrate (Aldrich) was used to determine the resulting inhibition of cystathionase activity.

Amino acid analysis of hydrolysates of native enzyme and of phenylglyoxal-treated enzyme revealed that only the level of arginine was modified by incubation with phenylglyoxal. As this investigation was directed mainly at determining the number of arginine residues involved in substrate binding, *S*-carboxyethylhomocysteine (*S*-CH), a competitive inhibitor of cystathionase [3,4], was added to the enzyme before incubation with phenylglyoxal and the number of arginine residues modified under those conditions was determined. A preliminary report of some of these findings has appeared [5].

2. Materials and methods

Cystathionase was prepared from rat liver as in [1]. The enzymic activities were measured as in [1] with the exception of the buffer used: indeed 125 mM bicarbonate buffer (pH 7.9) was employed as phenylglyoxal was dissolved in this buffer [6].

Lyophilised cystathionase (2 mg/ml) with phenylglyoxal (various concentrations) in bicarbonate buffer was incubated in the dark in capped tubes. When [^{14}C]phenylglyoxal was used, the incubated mixture was run on a column (1 × 16 cm) of Biogel P2 (Biorad) equilibrated with bicarbonate buffer. The protein peak was pooled and its radioactivity was determined in a liquid scintillation counter (Intertechnique) after mixing with Unisolve (Koch-Light). When unlabeled phenylglyoxal was used, the samples were occasionally run on a column of Biogel P2 or of Sephadex G-25 (Pharmacia) equilibrated with the bicarbonate buffer. Otherwise, at the end of the incubation, the samples were suitably diluted with bicarbonate buffer for measurement of enzymic activity or, to each sample (native cystathionase, phenylglyoxal-treated enzyme with or without preincubation with *S*-CH), an equal volume of 10% acetic acid was added. The mixtures were dialysed in the cold against 5% acetic acid and then 1% acetic acid, according to procedures employed for other enzymes [7,8]. After freeze drying each sample was hydrolysed for 24 h in vacuo at 110°C in 6 N HCl in presence of 20 μl or β-mercaptoacetic acid to prevent the regeneration of arginine during

hydrolysis [7]. The residue obtained after evaporation was dissolved in 0.1 N HCl. Amino acid analysis was performed on a Technicon Autoanalyser by the method in [9].

3. Results

We ascertained that the enzymic activities (L-homoserine deamination and L-cysteine desulfhydration) of cystathionase were, respectively, very similar when the measures were carried out in 125 mM bicarbonate buffer (pH 7.9) and in 0.2 M phosphate buffer (pH 7.8).

3.1. *Decrease of activities resulting from incubation with phenylglyoxal*

Incubation of cystathionase with phenylglyoxal (final conc. 1.7×10^{-3} M) at 4°C did not effect the activity whereas when the incubation was at 37°C the activity was significantly decreased. Table 1 shows the results of a typical experiment.

From the figures in table 1 it is obvious that incubation with phenylglyoxal produced a complete loss of both activities after 30 min and that phenylglyoxal, at the final conc., did not affect untreated enzyme. Similar results were obtained with 6.7×10^{-4} M phenylglyoxal which is the lowest concentration giving rise to complete inhibition of activity.

Table 1
Effect of phenylglyoxal on cystathionase enzymatic activity

Sample	Cysteine desulfhydration[a]		Homoserine deamination[b]	
1	29.7		249.2	
2	29.6		248.4	
3	4.6	84.5[c]	62.2	75[c]
4	n.d.	100[c]	n.d.	100[c]

[a] Expressed as μmol H_2S produced . h^{-1} . ml^{-1}
[b] Expressed as μmol α-cetobutyric acid produced . h^{-1} . ml^{-1}
[c] Percent of inhibition

(1) Enzyme; (2) enzyme added with phenylglyoxal (at the final concentration in the assay) without incubation; (3) enzyme incubated with phenylglyoxal (mixture final conc. 1.7×10^{-3} M) at 37°C for 15 min; (4) enzyme incubated in the same conditions than in 3 but for 30 min; n.d., not detectable. Incubation and activity measurement were in 125 mM bicarbonate buffer (pH 7.9)

These results reinforced the idea that arginine residues are components of the active center of cystathionase.

On the other hand, when cystathionase was incubated at 37°C with [^{14}C]phenylglyoxal, radioactivity was incorporated in the protein, which indicates that phenylglyoxal fixed on the protein was not eliminated by a run on a column of Biogel P2. This agrees with the observation that, for a sample of cystathionase incubated with unlabeled phenylglyoxal, enzymic activity was not recovered after a run on a Sephadex G-25 or of Biogel P2 column equilibrated with bicarbonate buffer.

3.2. *Amino acid analysis of hydrolysates of cystathionase*

The chromatograms revealed that the level of arginine was decreased in the phenylglyoxal-treated enzyme whereas no other amino acid was affected. In table 2, the number of arginine residues (calculated for mol. wt 180 000) recovered from hydrolysates of native cystathionase, enzyme incubated with phenylglyoxal (final conc. 6.7×10^{-4} M) with and without prior addition of *S*-CH (final conc. 5×10^{-3} M) are given. The number of lysine and histidine residues of the various samples are also given as these amino acids are particulary stable during acid hydrolysis.

The figures in table 2 also show that when the active center of cystathionase was 'protected' with *S*-CH against the effect of phenylglyoxal, 4 arginine/mol enzyme were not modified, which means that

Table 2
Number of some amino acid residues in native cystathionase and in phenylglyoxal-treated enzyme without and with preincubation with *S*-carboxyethylhomocysteine

Amino acid	Samples		
	1	2	3
Arg	45	18	22
Lys	83	83	83
His	35	35	36

The values, expressed as no. residues/mol cystathionase, are in every case the mean of 5 determinations. (1) Enzyme; (2) enzyme incubated with phenylglyoxal (6.7×10^{-4} M); (3) enzyme incubated with phenylglyoxal (6.7×10^{-4} M) after preincubation with *S*-CH (5×10^{-3} M)

they were prevented from reacting with phenylglyoxal. Indeed, over the 45 arginine residues calculated for native cystathionase, 18 were recovered in the phenylglyoxal-treated enzyme which indicates that 27 were modified, and 22 were recovered in the 'protected' enzyme, which indicates that 23 arginine residues were modified under those conditions.

4. Discussion and conclusion

The inhibition of cystathionase by phenylglyoxal provides additional support for the assumption that arginine residues are components of the active centre of rat liver cystathionase. Under the conditions chosen, phenylglyoxal reacts with arginine residues which appear as non-essential, or critical, for enzyme activity. Indeed, 27 arginine/mol enzyme were modified by phenylglyoxal while, in the presence of the competitive inhibitor *S*-CH, only 23 arginine reacted with phenylglyoxal. That is *S*-CH prevents the modification of 4 arginine/mol enzyme. This suggests that 4 arginine residues are essential for substrate binding. As it has been reported [10] and confirmed [11] that rat liver cystathionase is a tetramer, it is tempting to suggest that in each subunit 1 arginine residue is the binding point of the α-carboxyl group of the substrates.

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