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The Amino Acid Composition and Sulfur Distribution of Crystalline Rhodanese

BO SÖRBO

Medical Nobel Institute, Biochemical Department, Stockholm, Sweden

The amino acid composition of crystalline rhodanese has been determined by ion-exchange chromatography and found to be that of a simple protein. All the sulfur of the enzyme could be accounted for by cysteine, methionine and cyanide-labile sulfur.

The enzyme rhodanese has been crystallized from beef liver [1] and kidney [2] and shown to behave as a homogeneous protein in ultracentrifugation [1] and electrophoresis [1,3] experiments. It was previously [4] suggested that rhodanese contained an active disulfide bond, but this has recently [5] been disproved. Convincing evidence has been supplied by Westley and coworkers [6,7] for the rhodanese reaction mechanism being a double displacement with an enzyme-sulfur compound as an intermediate. The crystalline enzyme, as prepared by published procedure, is obtained in the form of this intermediate and thus contains labile sulfur, which is rapidly lost on incubation with cyanide or sulfite. Otherwise very little is known about the chemical composition of rhodanese. In the present communication amino acid analysis and determinations of total and cyanide-labile sulfur in the enzyme will be reported.

EXPERIMENTAL

Materials. Crystalline rhodanese was prepared from beef liver as previously [1] described. The specific activity of the preparation was 252 RU/mg (as defined in Ref.[1]) and its homogeneity was checked in the ultracentrifuge. The enzyme was freed from ammonium sulfate by dialysis against 0.01 M sodium acetate prior to amino acid analysis and by dialysis against 0.01 M ammonium acetate prior to sulfur analysis.

Amino acid analysis. The protein was hydrolyzed with 3 N HCl for 20 and 70 h in evacuated sealed tubes at 110°C. This HCl concentration was used instead of the usual 6 N HCl, as the latter was found to give considerably more humus formation without any improvement in extent of hydrolysis (as determined by the ninhydrin reaction [8]). The fact that 3 N HCl gave hydrolysates with welldefined, symmetrical peaks on amino acid analysis was confirmation of satisfactory hydrolysis. The hydrolysate was analyzed by automatic ion exchange chromatography as described by Moore *et al.*[9] The averages of the 20 and 70 h values were taken as representing the true content of each amino acid

except when the difference between the values was larger than 10 % of the mean. In these cases the values were either extrapolated to zero time of hydrolysis (where there was apparent destruction of the amino acid) or the 70 h value was used (where the amino acid was slowly liberated). The threonine and serine values were thus extrapolated to zero time of hydrolysis, assuming zero order kinetics for their destruction, as found by Smith *et al.*[10] in the case of papain. (However, Hirst *et al.*[11] found the destruction of these amino acids to follow first order kinetics in the hydrolysis of ribonuclease). In case of valine and isoleucine the 70 h values were taken to represent the true content of these amino acids.

Tryptophane was determined separately on the unhydrolyzed protein according to Bencze and Schmid.[12] Amide nitrogen was determined by a modification of the method of Laki *et al.*[13], the final determination of ammonia being done by the phenate-hypochlorite reaction.[14] For the determination of cystine/cysteine as cysteic acid, separate samples of the enzyme were oxidized with performic acid according to Pierce [15] and hydrolyzed as previously described. Cysteic acid was then determined in the hydrolysate by ion exchange chromatography.[9] As glutathione and oxidized glutathione gave 93.8 and 89.5 %, respectively, of the theoretical yield of cysteic acid when analyzed by this procedure, an average yield of 91.7 % was assumed for rhodanese. (Schram *et al.*[16] obtained a yield of 90 $\pm$ 2 % when cysteine, cystine and some proteins were oxidized with performic acid under similar conditions).

Sulfur analysis. Total sulfur was analyzed at the Microanalytical Laboratory, Department of Medical Chemistry, University of Uppsala.

Cyanide labile sulfur was determined by the following colorimetric method: About 1 mg of rhodanese (0.03 μmole) was incubated with 25 μmoles of KCN in a final volume of 0.5 ml for 5 min. The reaction was stopped by the addition of 0.5 ml 1 M acetic acid. (The same results were obtained, if the acid was added 1 min after the cyanide). The sample was then taken to dryness by evaporation at 60–80°C and the residue dissolved in 1 ml 0.5 M acetic acid and again taken to dryness. Thiocyanate was then determined in the residue by a modification [17] of the method of Boxer and Richards.[18]

RESULTS

Amino acid analysis of crystalline rhodanese gave the results shown in Table 1. As previous experiments [5,19] have shown the presence of 4 sulfhydryl groups in the rhodanese molecule, the values for cysteine/cystine obtained by cysteic acid analysis are given as cysteine. The complete amino acid analysis of a protein allows the partial specific volume of the latter to be calculated.[20] The data of Table 1 thus give a partial specific volume of 0.734 for rhodanese, which is in satisfactory agreement with the value 0.742 previously [1] obtained by pycnometric measurements. Rhodanese was found to contain 0.096 % cyanide labile sulfur, corresponding to 1.11 sulfur atom per enzyme molecule. The same value was obtained after the enzyme had been incubated with 0.03 M thiosulfate for 150 min and the excess thiosulfate then removed on a Dowex 2 (acetate) column. Bovine serum albumin, ribonuclease and egg albumin (all crystallized preparations) were also analyzed for cyanide-labile sulfur and found to contain 0.0045, 0.0037 and 0.0034 % labile sulfur, corresponding to 0.097, 0.015 and 0.048 atoms of sulfur per molecule. Whether these values just reflect an interference of protein in the analytical method or signify a small but significant content of labile sulfur in these proteins cannot yet be established. In any case, the results obtained for rhodanese demonstrated that the enzyme contained 1 labile sulfur atom per protein molecule. The total sulfur content of rhodanese was found to be 0.81 %, corresponding to 9.4 sulfur atoms per enzyme molecule. This is somewhat low in consideration of the results

Table 1. Amino acid composition of crystalline rhodanese.

Amino acid	Grams of amino acid residue per 100 g of protein			Calculated No. of residues [a]	Assumed No. of residues
	20 h	70 h	Average or extrapolated value		
Aspartic acid	7.49	7.34	7.42	23.9	24
Threonine	3.64	3.27	3.79	13.9	14
Serine	5.08	4.16	5.45	23.2	23
Glutamic acid	10.63	10.55	10.6	30.4	30
Proline	5.50	5.29	5.40	20.6	21
Glycine	4.36	4.19	4.27	27.8	28
Alanine	5.00	4.78	4.89	25.5	26
Valine	5.93	6.85	6.85	25.7	26
Methionine	1.90	1.84	1.87	5.29	5
Isoleucine	1.92	2.22	2.22	7.28	7
Leucine	7.94	8.23	8.09	26.5	27
Tyrosine	5.08	5.14	5.11	11.6	12
Phenylalanine	6.13	6.31	6.22	15.7	16
Lysine	5.86	5.41	5.64	16.3	16
Histidine	2.80	2.83	2.82	7.59	8
Arginine	8.90	8.95	8.93	21.2	21
Cysteine [b]	1.13	1.19	1.16	4.16	4
Tryptophane [c]	—	—	5.40	10.8	11
Ammonia [c,d]	—	—	0.686	15.9	16
Total	—	—	96.27	318.0	319

a Calculated for a molecular weight of 37 100.[1]
b Determined on performic acid oxidized protein as cysteic acid.
c Separately determined on unhydrolyzed protein.
d Omitted from total.

mentioned previously, which show that rhodanese contains 4 cysteine and 5 methionine residues (Table 1) together with 1 cyanide labile sulfur atom, making a total of 10 sulfur atoms per enzyme molecule.

DISCUSSION

The amino acid composition of rhodanese shows the picture of a simple protein. The fact that more than 95 % of the dry weight was accounted for by amino acid analysis indicates that there is no significant amount of unidentified constituents in rhodanese. The value obtained for cyanide-labile sulfur in the present investigation is lower than that reported by Westley and coworkers. The latter found 1.9 atoms of S/enzyme molecule using a polarographic technique [6] and 1.6 atoms of S/molecule when the enzyme was labelled with ^{35}S by incubation [7] with $^{35}SSO_3^{2-}$. They interpreted their results as demonstrating that rhodanese contains 2 labile sulfur atoms per molecule. However,

they did not report control experiments with other proteins and the possibility that their analytical techniques gave too high results cannot be excluded. But it is also possible that different rhodanese preparations may vary in their specific activity and content of labile sulfur due to alterations of the enzyme during the purification procedure. Such phenomena have been observed in case of the sulfhydryl content and specific activity of yeast alcohol dehydrogenase.[21] It is also possible, as mentioned by Westley and Nakamoto,[7] that two isoenzymes of rhodanese exist, one containing two and the other one labile sulfur atoms per enzyme molecule.

It should be pointed out that the labile sulfur found in crystalline rhodanese may be an artifact introduced during the purification, as thiosulfate is added in order to stabilize the enzyme. The labile sulfur is not necessary to the native protein, as claimed by Green and Westley.[6] It can be removed from the enzyme by a short incubation of fairly concentrated rhodanese with cyanide followed by removal of the latter on an anion exchange column, and an enzyme preparation thus treated still retains most of its activity (unpublished experiments).

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