ON THE SIZE AND CHEMICAL NATURE OF THE POLYPEPTIDE CHAIN OF BOVINE LIVER RHODANESE

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Received April 24,1975

Summary: Evidence from molecular weight studies and sequence analysis of bovine liver rhodanese indicates that the enzyme is a single polypeptide of molecular weight 35,200, and not a dimer of identical subunits half this size. The rhodanese molecule contains 317 amino acids including 5 methionines, 4 cysteines, and 5 tryptophans. As expected, six fragments were produced by cleavage with cyanogen bromide and these have been aligned in the enzyme with the aid of overlapping tryptic peptides isolated from a [^{14}C] carboxymethylmethionyl rhodanese derivative. The cyanogen bromide fragments account for all of the amino acid residues of the parent rhodanese molecule. Methionine residues are located at positions 72, 112, 214, 217, and 235 in the polypeptide chain and the active site cysteine is at position 251, in the carboxyl-terminal segment of the molecule.

In his early characterization of rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1), Sörbo (1) reported a molecular weight of 37,500 based upon sedimentation velocity-diffusion measurements. Subsequent studies, however, have led to the generally-held conception that native rhodanese is a dimer in rapid equilibrium with identical or similar monomers of molecular weight 18,000. Evidence in favor of this interpretation has been obtained from gel filtration and sedimentatic velocity studies (2), kinetic analysis (3), peptide mapping (2,4), and fluorescence polarization spectroscopy (5). Further support for the enzyme dimer has been derived from the fact that there appear to be two active sites per enzyme of molecular weight 36,000 (6,7,8,4). One serious objection to the proposed dimeric rhodanese structure has been the failure by numerous investigators to demonstrate dissociation of the enzyme into monomers under denaturing conditions; preparations so treated still yield molecular weight values in the neighborhood of 35,000 (4,9,10). Moreover, results obtained thus far in the x-ray crystallographic study of rhodanese at 4 Å resolution are difficult to reconcile with the dimer hypothesis (11). This communication presents definitive evidence, based primarily upon partial covalent structural analysis, that rhodanese is a single-chain polypeptide containing 317 amino acid residues and having a molecular weight of 35,200.

EXPERIMENTAL PROCEDURES

Molecular weight determination. Rhodanese was prepared as described earlier (4,12) and the molecular weights of 3-5 mg samples of the native enzyme and derivatives obtained by reduction and

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carboxymethylation (13,14) and performic acid oxidation (15) were determined by gel permeation chromatography under denaturing conditions as described by Fish et al. (16). The column was calibrated with similarly derivatized standards of known molecular weight (13).

Isolation of cyanogen bromide fragments. Reduced and carboxymethylated rhodanese (RCM-rhodanese) was treated with a two-fold excess by weight of cyanogen bromide (CNBr) as described earlier (17). Five fractions, designated CNBr-I to CNBr-V on the basis of their order of elution, were obtained by gel filtration of the reaction mixture on a column of Sephadex G-50 equilibrated and eluted with 50% acetic acid. Three of the fractions, CNBr-III, CNBr-IV, and CNBr-V were isolated in pure form by this single step. CNBr-I was purified from a mixture of partially-cleaved components by ion-exchange chromatography on a column of SP-Sephadex developed with buffers containing 8 M urea. CNBr-II was resolved by a similar procedure into two fractions, CNBr-IIA and CNBr-IIB.

Isolation of ^{14}C-carboxymethylmethionyl peptides from citraconylated RCM-rhodanese. To 10 ml of a solution containing 200 mg of RCM-rhodanese in 0.1 M sodium acetate, pH 4,0, and 6 M guanidine hydrochloride was added 0.5 ml of an aqueous solution of [2-^{14}C] iodoacetic acid (92 mg; 0.5 mmol; specific radioactivity, 40,000 cpm/μmole). After reaction for 24 hours in the dark, the solution was dialyzed against distilled water and lyophilized. The specific radioactivity of the product was 180,000 cpm/μmole, indicating the incorporation of 4.5 moles of substituent per mole of rhodanese (MW = 35,000). The [^{14}C]-carboxymethyl-methionyl-RCM-rhodanese was citraconylated (18) in 6 M guanidine hydrochloride, dialyzed against 1 mM N-ethylmorpholine acetate (NEMAC), pH 8.5, and lyophilized. Cleavages at arginyl residues were generated by hydrolysis with trypsin (1% by weight; TRTPCK-1FA, Worthington; 4 hrs, 37°) and the peptides were resolved into 13 fractions (TA-I to TA-XIII) by gel filtration on Sephadex G-50 in 0.1 M ammonium bicarbonate. All of the original radioactivity was recovered in fractions TA-I, TA-III, and TA-XIII. TA-I and TA-XIII were pure peptides containing a single residue each of labeled methionine. Ta-III was further resolved into two radioactive peptides, TA-IIIb and TA-IIIa, containing one and two residues, respectively, of [^{14}C]-carboxymethylmethionine. These tryptic fragments accounted for all of the 5 methionine residues in rhodanese.

Analytical procedures. Amino acid sequences of residues at the NH_2-termini of intact RCM-rhodanese, CNBr fragments, and tryptic peptides were derived by automated Edman degradation

(19) in a Beckman protein-peptide Sequencer (model 890-C) with dimethylallylamine protein and peptide programs supplied by the manufacturer. Phenylthiohydantoins were identified and quantitated by gas chromatography (20), high pressure liquid chromatography, and hydrolytic back conversion to amino acids (21). Thin layer methods (22), with or without ninhydrin staining (23), were employed for purposes of confirmation. Small peptides were sequenced manually (24).

RESULTS AND DISCUSSION

Molecular weight. In an earlier publication by Blumenthal and Heinrikson (4) the molecular weight of rhodanese, determined by gel filtration under nondenaturing conditions and polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) and mercaptoethanol (25), was reported to be 35,000. Essentially the same result is obtained when native rhodanese or derivatives thereof containing modified cysteinyl residues are subjected to gel filtration on columns of 6% agarose in the presence of 6 M guanidine hydrochloride and mercaptoethanol. These data are summarized in Table 1, together with molecular weight estimations of rhodanese made by various invesgiators employing these techniques as well as sedimentation methods. The evidence provided by these independent approaches indicates that the molecular weight of the enzyme is about 35,000 and that subunits, if present, must be linked by unconventional covalent bonds. Data presented below rule out this possibility.

Table 1

Molecular weights of rhodanese as determined by various procedures

Procedure	Molecular Weight
A. Gel Filtration	
1.) 6% agarose; 0.1 M sodium acetate 6 M guanidine HCl; 0.1 M mercaptoethanol (cf ref 16)	35,500 (native) 36,000 (RCM) 36,000 (oxidized)
2.) Sephadex G-75; 0.1 M Tris · SO_4, 0.01 M sodium thiosulfate, pH 8.0	35,000 (4)
3.) Sephadex G-100	19,000; 37,000 (2)
B. Polyacrylamide gel electrophoresis in SDS-mercaptoethanol (cf ref 25)	35,400 (4) 35,000 (9) 32,600 (10)
C. Sedimentation velocity	37,500 (1) 19,000; 38,000 (2)

Table 2

Amino acid composition of reduced and carboxymethylated rhodanese

Amino acid	Recovery[a]			Relative molar quantities[b]	No. of residues per molecule
	24 hrs	48 hrs	72 hrs		
		mμ moles			
Lysine	44.2	43.1	44.0	16.9	17
Histidine	21.3	20.8	19.2	7.9	8
Arginine	56.3	56.0	55.7	21.1	21
S-carboxymethyl Cysteine	9.3	8.5	8.0	3.5	4
Aspartic Acid + Asparagine	67.1	66.2	65.5	25.8	26
Threonine	31.2	28.0	22.2	13.9	14
Serine	50.0	31.7	20.8	20.7	21
Glutamic Acid+ Glutamine	86.3	81.3	82.0	32.2	32
Proline	52.5	51.1	52.4	20.2	20
Glycine	73.8	71.5	70.7	27.9	28
Alanine	66.9	65.9	67.8	26.0	26
Valine	69.4	69.5	72.1	27.3	27
Methionine	12.6	12.0	11.2	4.8	5
Isoleucine	20.6	19.8	20.8	7.9	8
Leucine	69.9	69.9	69.2	27.1	27
Tyrosine	24.9	18.3	13.9	11.7	12
Phenylalanine	41.2	40.4	40.8	15.7	16
Tryptophan[c]					5
Total					317

[a] Samples of 90 μg were hydrolyzed *in vacuo* in 6 N HCl at 110° for the times indicated.

[b] Calculated by dividing the corrected compositions by 2.58 mμ moles, the equivalent of 1 residue of amino acid. Based on Ala = 26.0 residues.

[c] Determined by hydrolysis in methanesulfonic acid (27) and by peptide analysis.

running time being extended to 7 hours for better separation. Molecular weight determinations of the major microsomal peptides by SDS-polyacrylamide[+] electrophoresis was also as previously described (15). The standards were serum albumine = 68,000, catalase = 60,000, ovalbumine = 43,000, and hemoglobin = 15,500. After staining with Comassie blue, the optical density of bands was measured with a Gillford scanning photometer at 610 nm.

Results: From Fig. 2 it is apparent that P-450 is most prominent in fraction II 30-35. This is not in agreement with earlier results of Lu and Levin (3), who reported that the P-450 richest fraction was obtained between 40-50 per cent $(NH_4)_2SO_4$, however, experiments with rat liver microsomes in our lab have failed to confirm these findings. The three other known components of microsomal electron transport NADH- and NADPH-cytochrome c reductase, and cytochrome b_5 are most prominently represented in fraction I 40-50. Preliminary results have shown that both type I and type II hydroxylase activities are present if a mixture of these two fractions, II 30-35 and I 40-50, is used for *in vitro* measurements; however, we feel that useful studies of recombined activity are dependent on a more detailed characterization of all peptides and lipids contained in these fractions. Separation of the proteins on SDS-polyacrylamide gels as shown in figure 3 revealed: (1) seven major components with molecular weights ranging from 10,000 to 100,000 daltons; (2) large quantitative and qualitative differences between ammoniumsulfate fractions within a given preparation; (3) quantitative differences in components for the same ammoniumsulfate fraction from variously treated animals. The largest difference seen in the variously treated groups was an increase in cytochrome P-450 content at elevated ammoniumsulfate concentrations after phenobarbital treatment.

By comparing quantitative functional measurements, seen in figure 2, with band intensity, we have assigned one major peptide to each of the 4 known components (Fig. 3-right). The assignment of activities to electrophoretic components as described in Fig. 4 was supported by a comparison of the ratio of similarly determined components, e.g. reductases or cytochromes, with

[+]SDS = sodium dodecylsulfate

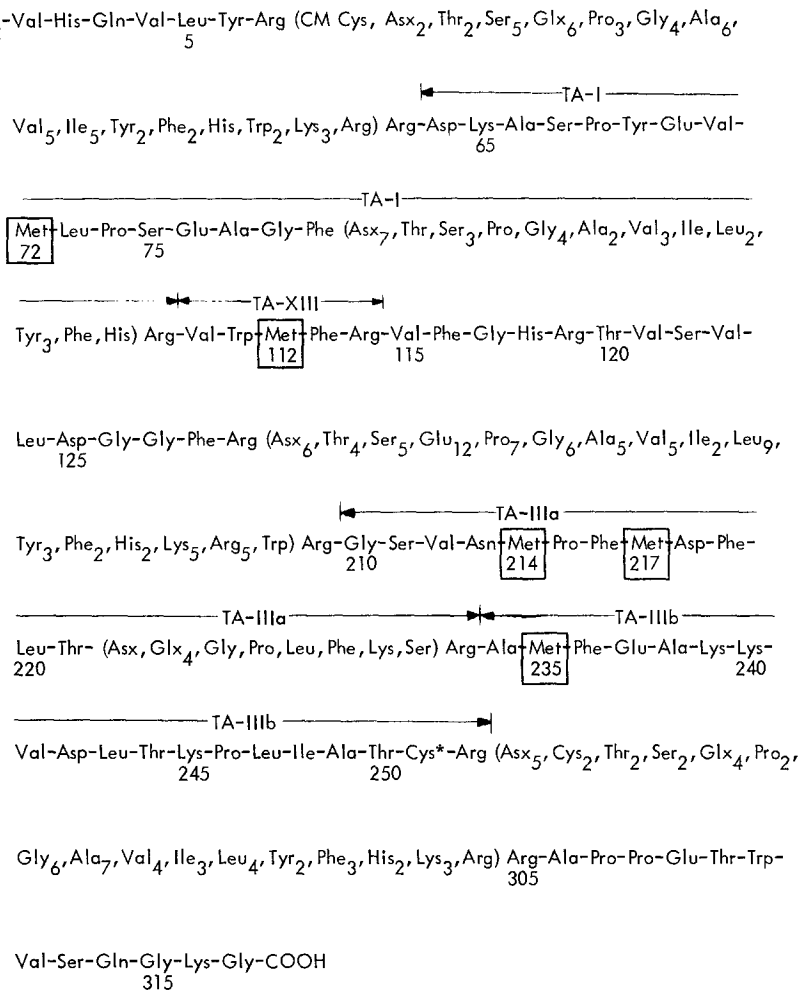

Fig. 1. Partial sequence analysis of bovine liver rhodanese indicating alignment of the six CNBr fragments. Arrows over each methionine residue indicate tryptic peptides from citraconylated [^{14}C]-carboxymethylmethionyl-RCM-rhodanese. Amino acids included within parentheses are based upon compositional analyses of each fragment minus residues placed in sequence. The latter assignments were made from automated Edman degradation of the fragments and carboxypeptidase Y digestion (see text).

TA-I: (residues 64-109). Sequence analysis of this peptide allowed for the placement of residues 64 through 79 and provided the overlap between CNBr-IIB and the next CNBr fragment, CNBr-III.

CNBr-III: (residues 73-112). Edman degradation proved the equivalence of positions 73-79 with the sequence in TA-I and established its location as the second CNBr fragment. COOH-analysis gave the sequence: -Val-Trp-Hse.

TA-XIII: (residues 110-114). The overlapping peptide between CNBr-III and CNBr-I gave the sequence: Val-Trp-Met-Phe-Arg. This is the fifth tryptophan peptide in rhodanese (cf. 28).

CNBr-I: (residues 113-214). Residues 113 through 129 were placed by Edman degradation and the COOH-terminal sequence of this 102-residue fragment was shown to be: -Val-Asn-Hse.

TA-IIIa: (residues 210-233). Amino acid analysis of this peptide revealed the presence of 2 residues of labeled methionine. Automated Edman degradation allowed the placement of residues 210-221, thus locating the fourth methionine at 217. This peptide contains CNBr-V, the tripeptide Pro-Phe-Hse, and provides the overlap into CNBr-IV, the penultimate CNBr fragment in rhodanese.

CNBr-IV: (residues 218-235). Sequence analysis of this fragment was in accord with its location between CNBr-V and the COOH-terminal peptide CNBr-IIA. The sequence of -Arg-Ala-Hse was identified at its COOH-terminus.

TA-IIIb: (residues 234-252). Automated Edman degradation proved that this fragment is that which contains the active site cysteine residue sequenced previously (14). The earlier analysis was in error and the corrected sequence in the vicinity of the active site sulfhydryl reported herein is in agreement with the structure found by Bossa et al. (30) for the tryptic peptide from bovine kidney rhodanese.

CNBr-IIA: (residues 236-317). The only CNBr fragment lacking Hse, this peptide is the COOH-terminal fragment in rhodanese. Its NH_2-terminal sequence is equivalent to residues 3-19 (236-252) in Ta-IIIb. The COOH-terminal sequence was obtained by analysis of a tryptic peptide from citraconylated material, the only peptide lacking in arginine. Most of this structure was reported earlier (28) in a study of the tryptophyl peptide sequences.

Thus, the total number of amino acids observed in the six cyanogen bromide fragments from rhodanese is equal to 317 residues, the number calculated from its composition (Table 2). One cysteine is located in the NH_2-terminal segment of the molecule and the remaining three cysteines, including the catalytically essential residue, are near the COOH-terminus. These findings constitute the first chemical proof for the existence of rhodanese as a single polypeptide chain the size of which is in accord with the majority of molecular weight studies of the enzyme. Moreover, they provide a structural framework for interpreting the results of x-ray crystallographic analysis and mechanistic studies of rhodanese currently underway in other laboratories.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the many helpful suggestions of Dr. John Westley throughout the course of these studies, and the expert technical assistance of Ms. Elaine Krueger. This work was supported by grant GB-29098 from the National Science Foundation

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