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A STUDY OF THE SINGLE POLYPEPTIDE NATURE OF RHODANESE A COMPARISON OF DIFFERENT PREPARATIONS

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Summary

The enzyme rhodanese (EC 2.8.1.1) appears as a single polypeptide chain protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of this species is approx. 33 000. This contrasts with previous reports that rhodanese behaves on gel filtration chromatography as a rapidly equilibrating monomer-dimer system composed of identical subunits with a molecular weight of 18 500.

We have investigated this apparent discrepancy by isolating the enzyme by the two different preparative procedures used in the above investigations. The two crystalline samples were subjected to gel filtration chromatography under a wide variety of conditions and to sodium dodecyl sulfate disc gel electrophoresis.

The two preparations yielded rhodanese which behaved identically and no evidence for the monomeric species was obtained under any experimental condition tested.

Thin-layer gel chromatography of clarified liver homogenates gave no evidence of rhodanese species other than that present in the purified samples.

The variation in molecular weights observed in gel filtration chromatography may be a reflection of the conformational mobility of the enzyme leading to solvent-dependent changes in Stokes radius. If rhodanese is dimeric, special interactions must stabilize it under the conditions tested here.

Introduction

Early sedimentation studies showed that the enzyme rhodanese (thiosulfate : cyanide sulfurtransferase, EC 2.8.1.1) behaved in a way expected if its molecular weight was 37 000 [1]. Subsequent investigations were taken to indicate that rhodanese was a dimer composed of two identical or nearly identical subunits of molecular weight 18 500. These subunits were in a rapid, pH-depen-

dent equilibrium with the dimer [2]. The primary pieces of evidence for this interpretation were the elution patterns from Sephadex G-100 columns and the results of detailed sedimentation studies. At pH 7.0 the rhodanese pattern was characteristic of a rapid monomer-dimer equilibrium [3], but at pH 9.0 the pattern was that of a non-dissociating system of molecular weight 18 500. When rhodanese was stored in air at 0—2°C a dimeric species of molecular weight 37 000, linked by what appeared to be disulfide bonds, could be partially separated from the monomer at pH 9.0. Sedimentation studies of rhodanese also revealed this time-dependent molecular weight change from 18 500 to 37 000 under conditions favorable to sulfhydryl group oxidation [2]. This description of the structure of rhodanese was supported by tryptic peptide maps [2], fluorescence spectroscopy [4], and labelling studies which demonstrated the existence of two active catalytic sites per molecular weight 37 000 [5].

More recently, however, this description of rhodanese has been the subject of reexamination, with evidence accumulating to indicate that rhodanese is a single, non-dissociating protein of molecular weight closer to 35 000. Such evidence has been obtained notably from amino acid sequencing [6], and the repeated failure of investigators to detect a lower molecular weight species under denaturing conditions [7—9].

The more recent studies, however, have not excluded the existence of the dimeric form of rhodanese. First, the original investigations isolated pure active rhodanese by the method of Westley and co-workers [10,11], whereas the more recent investigations used rhodanese as isolated by Horowitz and DeToma [12] or a modified form thereof [7]. Secondly, it is not known whether rhodanese exhibits anomalous behavior under the denaturing conditions used by recent investigators in their molecular weight determinations [7—9]. In light of these considerations, we have compared the rhodanese prepared by the two methods, in the hope of understanding the divergent observations of the molecular weight of this enzyme.

Materials and Methods

Bovine liver rhodanese was prepared both by the method of Davidson and Westley [11,10], and by the method of Horowitz and DeToma [12]. Enzyme activity was measured as previously described [1,5]. Protein concentrations were determined with a modified biuret method [13]. Enzyme with a specific activity of 0.71 I.U./μg is taken to be 100%.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate discontinuous polyacrylamide gel electrophoresis samples were prepared and run by the method of Maizel [14], and analyzed according to Weber and Osborn [15]. The standards employed were bovine serum albumin, ovalbumin, chymotrypsinogen, and myoglobin, of molecular weights 65 000, 45 000, 25 000, and 16 900, respectively.

Sephadex gel chromatography. Sephadex G-75 was hydrated for 6 h at 100°C, followed by stirring and decantation to remove bead fragments. All column packing and chromatography was done at 4°C. The equilibration and elution buffers were 0.05 M phosphate, 0.05 M borate at pH 7.0 or 9.0. All

protein samples were dissolved in these buffers. All fractions were 0.19 ml. Two columns of Sephadex G-75 were used. The first (1.6 × 32.8 cm) was calibrated with β-lactoglobulin and chymotrypsinogen (1.0 ml of 5.0 mg/ml each) at pH 7.0, and rhodanese prepared by the method of Horowitz and DeToma [12] (to be designated Rhodanese II) was chromatographed at pH 7.0. The second column was calibrated with bovine serum albumin, β-lactoglobulin, chymotrypsinogen, and myoglobin at pH 7.0, and rhodanese II was chromatographed at the same pH. The column expanded to 1.6 × 24.7 cm when the buffer was changed to pH 9.0, and was recalibrated with β-lactoglobulin and myoglobin following runs with rhodanese II at pH 9.0, Davidson-Westley rhodanese at pH 9.0 (to be designated rhodanese I), and four runs with rhodanese I at pH 7.0. All flow rates except for the final run were 0.3 ml/min. The final run was 0.03 ml/min.

Fractions collected were assayed for enzyme activity [5]. Protein concentrations from chromatography of the calibration standards were determined by the biuret method [13].

Thin-layer gel chromatography. Sephadex G-75 superfine was hydrated at 100°C for 3 h, and a layer 1.0 mm thick was spread on a 20 × 40 cm plate. Plates were equilibrated overnight with 0.1 M Tris · HCl buffer at pH 7.9. Samples containing 0.5—10 μg of rhodanese in 10 μl were applied to the plate. Protein standards contained 100 μg in 10 μl. Blue dextran 2000 was used as a marker. Following runs at 15° slope for approx. 6 h, the plate was replicated with Whatman 3 MM paper [16,17]. To develop for rhodanese activity, the paper was sprayed using an atomizer with 5 ml 0.040 M KH_2PO_4, 0.050 M KCN, 0.050 M $Na_2S_2O_3$. Following incubation for 1 h at 25°C in a saturated atmosphere, the paper was sprayed with 5 ml 18.5% formaldehyde, followed by 5 ml 0.165 M $Fe(NO_3)_3 \cdot 9H_2O$, 13.3% HNO_3. The rhodanese activity appeared as an elongate brown spot 3—5 cm long. The paper was then fixed and developed for protein content by soaking for 1 h in Coomassie blue solution (2.5% Coomassie blue in acetic acid/methanol (10 : 90, v/v)), and destained for 3 h in methanol/acetic acid/water (50 : 10 : 40, v/v). R_F values were calculated relative to ovalbumin, using as the migration distance the center of the elliptical spot. The protein standards used were ovalbumin, chymotrypsinogen, and myoglobin. Molecular weight determinations were made by plotting $1/R_F$ versus log molecular weight [17].

Results

Identity of enzyme preparations

Rhodanese I and rhodanese II (see above) with specific activities greater than 90% were prepared via both preparations, and subjected to comparison.

Sodium dodecyl sulfate disc gel electrophoresis. Rhodanese II from two different preparations was run versus protein standards and gave a single homogeneous band at 33 200 in all samples. Rhodanese I samples of purity 63, 89, and 100% obtained from the three final steps of the preparation, respectively, were similarly tested. The three samples migrated distances virtually identical to that of a rhodanese II sample run at the same time, corresponding to molecular weights of 32 600. Impurities in the 89% sample were detected at

distances corresponding to molecular weights of 30 100 and 57 300, and in the 63% sample at 44 500 and 52 300. In no rhodanese gel, including several overloaded gels, was there a detectable band below molecular weight 30 000.

Sephadex chromatography at pH 9.0. In successive runs on the second column (see above), rhodanese I and rhodanese II, both derived from 100% crystals, were chromatographed. The elution volumes were 24.05 and 24.00 ml, respectively, with symmetrical elution peaks in both. Biuret assays on fractions collected in the run with rhodanese II duplicated the activity peak, with no protein detectable at an elution volume corresponding to molecular weight 18 500 (30.05 ml).

Absence of a monomeric form of rhodanese

Gel filtration studies were conducted to maximize the possibility of detecting any monomeric form. In all runs the rhodanese was applied to the column within 2 h of thawing from storage at −70° C in a 1.0 M $(NH_4)_2SO_4$ crystalline suspension, with the exception of the pH 9.0 run of rhodanese I, where the interval was 4 h. The time difference was not significant since the pH 9.0 runs of rhodanese I and II gave identical elution volumes. The chromatography runs took an average of 2 h, and assays were conducted immediately upon completion of the run. The sample volumes and several sample protein concentrations used were considerably less than those used in previous studies [2], thus favoring the equilibrium formation of monomeric 18 500 molecular weight form. In addition, estimations of molecular weights corresponding to elution peaks were more reliable since the sample volumes were small compared to the elution

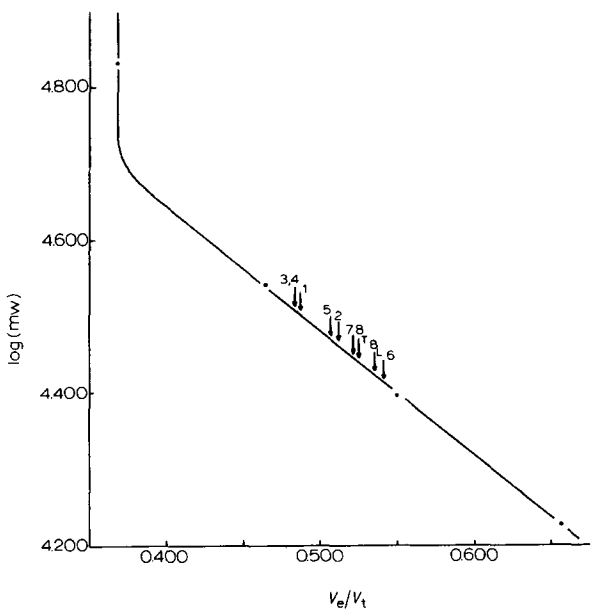

Fig. 1. Results of Sephadex chromatography of rhodanese. Arrows indicate elution peaks of the various runs conducted with rhodanese. Numerals refer to the runs tabulated in Table I. Standards (•) are (left to right): bovine serum albumin (excluded); β-lactoglobulin; chymotrypsinogen; myoglobin.

TABLE I

RESULTS OF SEPHADEX CHROMATOGRAPHY RUNS ON RHODANESE

Run	Sample	pH	V_e/V_t	Mol. wt.
1	Rhodanese II (2.7 mg/1.23 ml)	7.0	0.487	32200
2	Rhodanese II (0.43 mg/0.45 ml)	7.0	0.512	29300
3	Rhodanese II (0.85 mg/0.50 ml)	9.0	0.483	32600
4	Rhodanese I (0.38 mg/0.70 ml)	9.0	0.483	32600
5	Rhodanese I (1.7 mg/0.78 ml)	7.0	0.507	29800
6	Rhodanese I (2.7 mg/1.18 ml)	7.0	0.542	26000
7	Rhodanese I (0.24 mg/0.68 ml)	7.0	0.522	28000
8	Rhodanese I (9.9 mg/18.0 ml)	7.0	0.535 Leading	26800
			0.525 Trailing	27800

volumes. Molecular weight estimates were made according to Andrews [18,19], and are shown in Fig. 1 and tabulated in Table I.

At pH 9.0 the elution peaks for both rhodanese I and II were symmetrical and centered at identical elution volumes. The molecular weight corresponding to these peaks was determined to be 32 600. In gel filtration runs at pH 7.0 we were unable to reproduce the elution patterns as described previously [2], but obtained instead symmetrical peaks with elution volumes corresponding to molecular weights ranging from 26 000 to 32 200. A typical elution pattern is shown in Fig. 2.

Sodium dodecyl sulfate disc gel electrophoresis was run using rhodanese samples which were taken from fractions eluted from the column in three different runs on rhodanese I at pH 7.0 (Runs 5, 6 and 7 of Table I). A comparison between samples taken from halfway up the leading boundary with samples taken from halfway down the trailing boundary should reveal whether

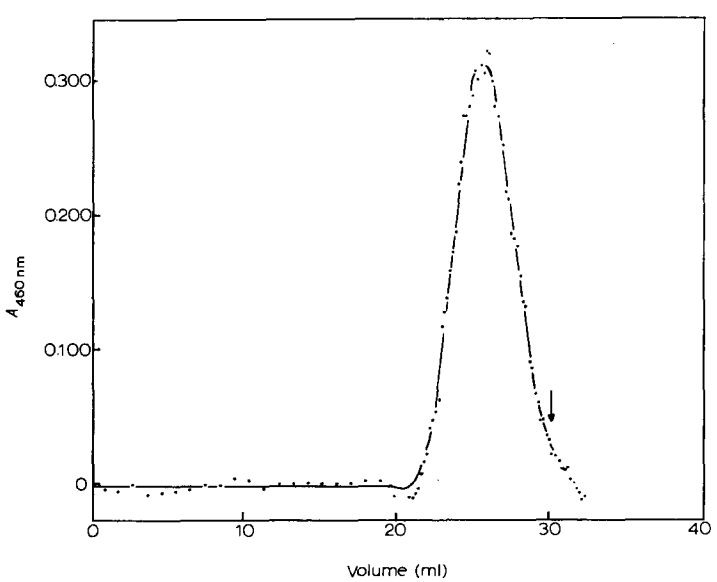

Fig. 2. Chromatography of rhodanese run No. 5: rhodanese I, 1.7 mg in 0.78 ml at pH 7.0. Arrow indicates expected elution volume of a molecular weight 19 000 species.

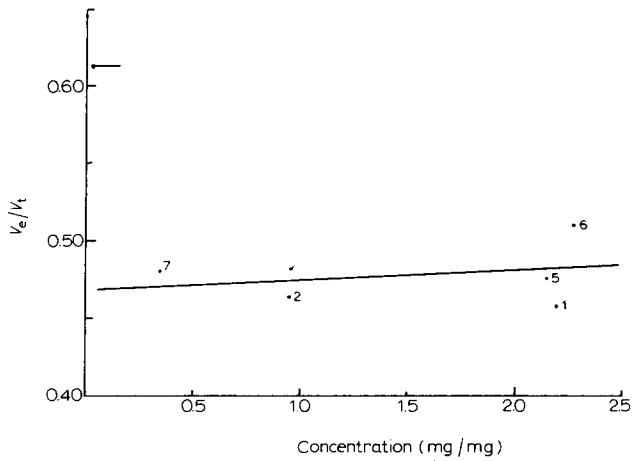

Fig. 3. Dependence of position of advancing boundary on sample rhodanese concentration. V_e/V_t was calculated from elution volume of the fraction located halfway up the leading boundary concentration gradient. Arrow on ordinate axis indicates expected elution position of a molecular weight 19 000 monomer.

any amount of rhodanese with a polypeptide chain of molecular weight 18 500 had been fractionated on the column. Secondly, the pH values of three different fractions taken from the trailing boundary of one run at pH 7 (Run 6, Table I) were adjusted to 9.0, 9.6, and 10.6 and the fractions were incubated for 3 h. In this manner we further favored the appearance of the monomeric form. The protein was denatured at these new pH values and electrophoresed. In every protein sample tested, both from leading and trailing boundaries, and from the pH-adjusted samples, single, sharp protein bands appeared at molecu-

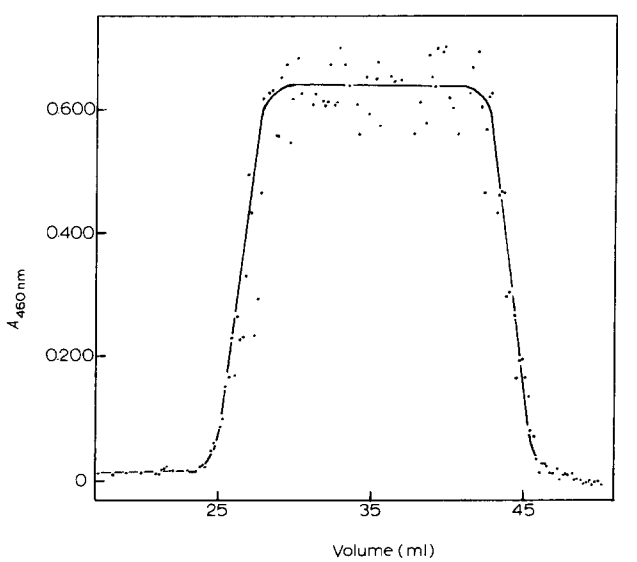

Fig. 4. Elution profile of rhodanese I run No. 8. 9.9 mg in 18.0 ml, pH 9.0.

lar weights ranging from 31 800 to 34 000. In no gel were bands detected below a molecular weight of 31 800.

In a further analysis for the presence of the monomeric form, the position of the advancing boundary was compared with the protein concentration of the applied sample. With a single non-dissociating species the position should be independent of sample protein concentration [3]. In fig. 3 the concentration dependence of the position of the advancing boundary is plotted and it appears that we are seeing the behavior of a single, non-dissociating species.

Our final chromatographic run of rhodanese I at pH 7.0 used a sample of approximately the same relative volume as that used by Volini et al. [2], giving a plateau in the elution diagram, but with a lower sample rhodanese concentration to favor the monomeric form. This run was performed to check the possibility that the smaller sample volumes used in previous runs failed to reveal the monomer-dimer equilibrium. We reduced the flow rate of our column to one-tenth that used previously [2] to ensure equilibrium sieving on the column. Under these conditions a monomer-dimer equilibrium would be detectable by a diffuse trailing boundary. Instead, a symmetrical elution pattern was obtained as shown in Fig. 4. Molecular weight estimation from the positions of the leading and trailing boundaries [19] gave the molecular weights of 26 800 and 27 800. That the latter molecular weight is higher reflects a slight hypersharpening of the trailing boundary.

Rhodanese in the liver extract

To investigate the possibility of a monomeric form of rhodanese existing in the original liver extract, thin-layer gel chromatography was performed at pH 7.9 using the crude liver extract, following centrifugation to remove insoluble residue. Although the sieving processes cannot be well characterized in this experiment due to the high protein concentrations (93 mg/ml) needed to ensure detection of active rhodanese (0.05 mg/ml), the location and spreading of the activity of rhodanese could be compared with that of purified rhodanese II. Both the purified rhodanese and the rhodanese activity spot in the liver extract were located at the same migration distance with the same degree of spreading (4.5 cm on a 40 cm plate). The estimated molecular weight was 27 000. This indicates that active rhodanese in the crude extract behaves as the same molecular weight species isolated in both preparations.

Discussion

In the dimeric model of rhodanese, the enzyme is composed of two identical subunits of molecular weight 18 500 that are in a rapid, pH-dependent equilibrium with the active dimer. At pH 9.0 the predominant species is the monomeric form. Each monomer contains two cysteine residues, one of which is located at the active site. At pH 7.0 the monomer is in equilibrium with the dimer of molecular weight 37 000. Under mild oxidizing conditions the non-active site cysteines can form a disulfide bridge resulting in a stable, active dimer. In the presence of the monomer this active dimer is inactivated by sulfhydryl-disulfide interchange involving the active site cysteine [2,4,20,21].

In the series of experiments described here, we searched for evidence of this dimeric equilibrium. Evidence for the monomer-dimer would have been reproduction of the characteristic gel chromatography results seen previously [2], or demonstration of the presence of a polypeptide of molecular weight of approx. 19 000 under denaturing conditions. Our first step was to determine whether the rhodanese isolated by the Davidson-Westley preparation [10,11] was the same as that by the Horowitz-DeToma preparation [12]. In molecular weight tests that sieved the protein both by the length of the denatured polypeptide chain and by its Stokes radius, the native protein from both sources behaved identically with a molecular weight of 32 600 (sodium dodecyl sulfate disc gel electrophoresis). This finding was further supported by evidence that active rhodanese in the crude liver extract behaves the same as that isolated by either preparation.

Allowing that the dimeric rhodanese described previously may have been present in small proportions efforts were made to produce a dissociated species under favorable conditions. Our experiments, however, gave no evidence of the existence of a monomeric form of molecular weight 18 500. Symmetrical peaks were obtained in every chromatographic run conducted, including a pH 7.0 run under experimental conditions similar to the run at pH 7.0 of Volini et al. [2]. All estimated molecular weights were greater than 26 000, especially those made at pH 9.0. The position of the advancing boundary was independent of the rhodanese concentration of the applied sample which is characteristic of a single species. The variation in molecular weights obtained at pH 7.0 was shown not to be due to the presence of a molecular weight 18 500 monomer by sodium dodecyl sulfate disc gel electrophoresis on fractions eluted from the column. The protein eluted from the column behaved as a species with a molecular weight greater than 31 800 when denatured and electrophoresed, regardless of whether the sample was taken from early in the elution diagram or late, or denatured at pH 7.0, 9.0, 9.6, or 10.6. Further, no molecular weight species lower than 30 100 was detected in impure rhodanese samples taken from the later stages of a preparation similar to that used previously [2].

The reasons for the discrepancies between our gel filtration results and those of Volini et al. [2] are not evident. A discussion by Ellis and Woodward [9] offers a possible reason. Since rhodanese is eluted in the latter portion of the elution diagram of Sephadex G-100 non-equilibrium sieving and zone broadening can occur, especially if flow rates are too great. Secondly, molecular weight estimations are risky when large sample volumes relative to the elution volume are used [9,22]. Possible non-equilibrium sieving was reduced in our run with a large sample volume by using a flow rate of 0.03 ml/min.

Sephadex gel chromatography sieves proteins on the basis of their Stokes radius [23]. It is tempting to speculate that the variability of the molecular weights obtained at pH 7.0 is a reflection of Stokes radius differences in the rhodanese chromatographed, since it was shown not to be due to differences in polypeptide chain length by subsequent denaturation and electrophoretic analysis.

It is interesting that rhodanese has been shown to be a conformationally mobile protein and its solution structure depends on the details of the environment [21,24]. The exact solvent conditions by affecting the tertiary structure

of the enzyme may partially account for observed discrepencies in the gel filtration behavior of this enzyme.

It is difficult to reconcile the results presented here with a number of previous studies which are consistent with the monomeric form of rhodanese [2,4,5,20]. However, if the enzyme can be dissociated, the conditions favorable for the production of monomer have not been found in this investigation and special factors stabilizing the dimer must be invoked, although these factors do not appear to affect the retardation coefficient or free electrophoretic mobility of the enzyme [9]. One possibility may be in the recent elegant demonstration of stoichiometric metal ion binding by rhodanese [25]. This metal chelation could strongly affect the quaternary structure of the enzyme and more specific denaturing conditions may be required. However, it should be noted that recent X-ray crystallographic studies of rhodanese (rhodanese II) are clearly consistent with the single polypeptide chain picture of this enzyme [26].

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