ACTA CHEMICA SCANDINAVICA 5 (1951) 724–734

On the Properties of Rhodanese

Partial Purification, Inhibitors and Intracellular Distribution

BO H. SÖRBO

Biochemical Department, Medical Nobel Institute, Stockholm, Sweden

In 1932, K. Lang described an enzyme, that in the presence of a suitable sulfur donor was able to convert cyanide to thiocyanate. As sulfur donors only thiosulfate and colloidal sulfur were found to be effective. The enzyme was named rhodanese and found to be present in high amounts in liver tissue from different animals. Lang also tried to purify the enzyme and obtained a 20-fold purification. Cosby and Sumner [2] reported a 100-fold purification from beef liver, but without giving any specific activity data for their purified material. Here will be described a method for purification of rhodanese which gives preparations about 150-fold more pure than the starting material (beef liver) and about 5 times more active than preparations obtained according to Cosby and Sumner. In connection with the purification experiments, the intracellular distribution of the enzyme has been studied.

METHODS

The rhodanese activity was assayed by a modification of Cosby and Sumners procedure. The system consisted of 2 ml of substrate pH 7.4 containing 0.05 *M* cyanide, 0.05 *M* thiosulfate and 0.05 *M* phosphate, and 0.5 ml of the rhodanese solution. The time of reaction was 5 minutes and the temperature 20°. The reaction was stopped by adding 2.5 ml of ferric nitrate-nitric acid [2], and the test sample was diluted to 30 ml and filtered if necessary. 30 minutes after the addition of ferric nitrate, the extinction at 460 mμ was determined in the Beckman spectrophotometer and the thiocyanate formed was obtained from a standard graph. One rhodanese unit (RU) was taken as the amount

* A preliminary report of this work was presented before the XVIII International Physiological Congress, Copenhagen, 1950.

of enzyme which under the given conditions produced 10 μ equivalents of thiocyanate. The specific activity for the different preparations is expressed as rhodanese units per mg protein, the latter determined by the turbidimetric method of Bücher [3] in the purification experiments and obtained from nitrogen determinations in the distribution studies. In the inhibition studies the enzyme was incubated with a solution of the inhibitor adjusted to pH 7.4 and containing 0.01 *M* phosphate. 30 minutes after adding the enzyme the activity was determined in the usual way. In a control test the enzyme was incubated with 0.01 *M* phosphate buffer pH 7.4.

In the enzyme distribution studies a modification of the method of Hogeboom, Schneider and Pallade [4, 5] was used. Adult guinea pigs were killed by exsanguination and the liver removed and chilled in cracked ice. All the operations during the fractionation were carried out at + 4°. A sample of liver was homogenized in 9 volumes of 30 % sucrose, and the homogenate centrifuged for 10 minutes at 1 100 g. The sediment was washed once by resuspending in half the original volume of sucrose and recentrifuging at the same speed, and the final sediment was suspended in 30 % surcrose. The supernatants were combined and centrifuged for 20 minutes at 19 000 g, to sediment the mitochondria. The sediment was washed twice by resuspending in half the original volume of sucrose and resedimented as before. The mitochondria were finally suspended in 30 % sucrose and the supernatants were combined. Since recent work [6] has indicated that it is possible that "microsomes" are artifacts obtained from mitochondria during the homogenization, the separation of "microsomes" from the supernatant was not undertaken. All of the activity determinations on these fractions and the parent homogenate were carried out at the same time after dilution with 30 % sucrose.

PURIFICATION OF ENZYME

In preliminary experiments different tissues from different animals were assayed for activity. As beef liver was most active, we decided to use it as the starting material. The results are shown in Table 1.

Extraction: Different extraction procedures were tried. The best one was as follows: The liver (fresh or frozen material gave the same results) was disintegrated in a Turmix blendor with 2.5 volumes of water, and 0.2 volumes of a 20 % lead acetate solution was added. When the suspension was centrifuged, a clear supernatant was obtained. If the lead acetate addition was omitted, turbid extracts with the same activity were obtained. However, only about 55 % of the total activity in the liver was extracted, the rest seemed to be bound to insoluble particles. No improvement in yield was obtained by

Table 1. Rhodanese activity for different tissues.

Tissue	Activity in RU/g fresh weight
Beef liver	81
» »	99
» »	76
» kidney	27
Horse liver	32
» kidney	20
Sheep liver	39
Guinea pig liver	55
» » »	41
» » »	52
Salivary gland from beef	3.1

using a larger volume of water, by repeated extractions of the precipitate or by using salt solutions as extracting solvents. Mincing the liver in an ordinary meat mincer gave much smaller yields than homogenizing it in the Turmix blendor. The extraction procedure given by Cosby and Sumner gave only about 50 % of the yield and purity obtained by our procedure. They denaturated the hemoglobin in their extracts with ethanol and chloroform according to Tsuchihashi, but since this procedure seemed to modify rhodanese and since it was possible to obtain preparations essentially free from hemoglobin in other ways, we avoided this step. Autolysis of liver brei in the presence of ethyl acetate destroyed the enzyme, but active autolysates could be prepared by the aid of chloroform or toluene, however with no improvement in yield or purity.

Ammonium sulfate fractionations: When ammonium sulfate fractionation of the extract was tried, it was found that when the fractionation was carried out at pH 5 or higher, the purificatic n was only 3-fold, but when the pH was lowered a much better result was obtained. At a lower pH more of the inert proteins were denatured and precipitated on the addition of ammonium sulfate to 10 % saturation. It was necessary, however, to separate this precipitate from the solution before increasing the salt concentration further, otherwise it carried down a considerable part of the activity. The optimum purification was obtained at pH 3.8; at this pH the fractionation must be carried out in the cold room. The enzyme was precipitated between 40 and 55 % saturation of ammonium sulfate and the precipitate was dissolved in a 0.05 *M* solution of sodium thiosulfate and Na_2HPO_4 to a concentration of 120 RU per ml. To it was added 1.3 volumes of 80 % saturated ammonium sulfate solution, brought to pH 8.1 with ammonia. The inactive precipitate was centrifuged off and discarded, and the enzyme precipitated from the supernatant by addition of

25 g ammonium sulfate per 100 ml original solution. The precipitate was dissolved in distilled water.

Acetone fractionation. After dialysing the ammonium sulfate fractionated enzyme against distilled water until free from salt, fractionation with organic solvents was tried. It was found that ethanol fractionation gave poor yields but methanol or acetone gave better results. To the dialysed enzyme solution was added 0.05 volumes of 0.1 *M* acetate buffer pH 4.63 and acetone to 35 % by volume. The inactive precipitate was centrifuged off and discarded, and the enzyme precipitated by raising the acetone concentration in the supernatant to 50 %. All these operations were carried out at 0° to — 5°. The precipitate now contained rhodanese purified 50-fold from the first extract and about 150-fold from the liver. The results from a typical purification experiment are shown in Table 2.

Table 2. Purification of rhodanese.

Preparation	Total activity RU	Specific activity RU/mg protein
Beef liver	152 000	0.25
Lead acetate extract	86 600	0.82
First ammonium sulfate ppt	35 200	12.6
Second » » »	30 200	25.4
After dialysis	24 400	27.2
Acetone ppt	12 800	41.8

Properties of purified material. A 1 % solution of the purified material was nearly colorless, only a faint yellow tinge attributable to traces of hemoglobin remained. The absorption spectrum determined in the Beckman spectrophotometer showed only the usual protein band at 2 800 Å and a very low Soret band. No change in the spectrum could be detected after adding thiosulfate to 0.01 *M* concentration. Electrophoresis in the Tiselius apparatus at pH 5.4 showed 4 different fractions, 2 moving toward the anode and 2 moving toward the cathode. At this pH the rhodanese moved toward the anode. The two anodically migrating peaks made up about 30 % of the total area, which indicated that the enzyme was far from pure. The iso-electric point of the enzyme must be lower than pH 5.4, therefore attempts to carry out electrophoresis at a lower pH were made. They proved however unsuccessful, as the enzyme was inactivated during the dialysis.

STABILITY OF RHODANESE

Experiments on the heat stability of rhodanese were carried out with the crude extract. It was found that the enzyme could withstand 45° for 5 minutes without any inactivation. In the presence of thiosulfate the stability was extended to 55°. Only small amounts of foreign proteins were however denaturated below these temperatures. The results are shown in Table 3.

Table 3. Heat stability of rhodanese *.

Temperature (°)	Remaining activity in % Without thiosulfate	In 0.01 *M* thiosulfate
40	99	—
45	90	96
50	63	92
55	4	87
60	0	1

* Lead acetate treated extract from beef liver heated for 5 min. at the indicated temperature

The enzyme in the crude extract was stable for 30 min. at room temperature from pH 4.5 to pH 10.0, while pH 4.0 and 10.5 each gave about 40 % inactivation. In the cold room (+ 4°) the stability was extended down to pH 3.5. The crude extract could stand dialysis against distilled water without inactivation. With the most purified preparations, however, a prolonged dialysis gave a loss in activity, which was always accompanied by a decrease in pH and a precipitate in the dialysis bag. The activity of different rhodanese preparations was not increased by the addition of boiled preparations of the enzyme or of preparations inactivated at a high or low pH. Attempts to split off any prosthetic group by precipitating the enzyme with ammonium sulfate at an acid or basic pH were unsuccessful. Hence no evidence for the presence of a dissociable prosthetic group in rhodanese was obtained.

INHIBITORS

The effect of different enzyme inhibitors was studied on a partly purified preparation.

Metal enzyme inhibitors: The only compound found to be active was cyanide [7] as shown in Table 4 a. Cyanate and acetonitrile gave no inhibition. The possibility that the enzyme contains an active metal group, which is inhibited by cyanide, cannot be excluded. However the lack of inhibition with

other metal enzyme inhibitors makes this assumption more unlikely. This is in contrast to the results recently published by Saunders and Himwich [8]. They found rhodanese to be inhibited by cysteine and sulfide and concluded that rhodanese is a metal enzyme. The discrepancy between their results and ours may be due to the different conditions used in the test system. It has also been claimed [9] that rhodanese is inhibited by taurocholate, which was explained as due to a reaction between taurocholate and an iron containing active group in the enzyme. The inhibition obtained with taurocholate in our experiments can, however, be explained by the denaturating effect of this compound on proteins in general [10].

Carbonyl group reagents: Cyanide is also known to react with carbonyl groups and the effect of carbonyl group reagents on rhodanese was therefore studied, Table 4 b. Sulfite and hydroxylamine gave a strong inhibition, while semicarbazide and phenylhydrazine were less active and thiosemicarbazide gave no inhibition at all. It has been shown [11, 12] that phenylhydrazine can inactivate sulfhydryl enzymes by oxidizing their sulfhydryl groups. When rhodanese was incubated with the carbonyl group reagent in the presence of cysteine, no inhibition was in fact obtained. The lack of inhibition with thiosemicarbazide, which itself contains a sulfhydryl group, is also significant. The presence of an active carbonyl group in the enzyme could thus be excluded. But also the inhibition with cyanide could be prevented by the presence of a tenfold excess of cysteine or thiourea. That the inhibition of rhodanese with cyanide could be prevented by cystine, was recently shown by Saunders and Himwich [8]. Cyanide reacts however with cystine under formation of thiocyanoalanine and cysteine [13]. Hence cyanide was removed from their system and cysteine formed and the experimental conditions thus not so well chosen. In our experiments the cysteine was dissolved in the presence of cyanide in order to prevent any formation of cystine. Also the sulfite inhibition could be partially prevented by cysteine or thiourea. The protecting effects of these compounds against cyanide or sulfite inhibition is difficult to explain, as cyanide and sulfite do not inhibit but on the contrary activate sulfhydryl enzymes. Lang has already shown [7] that cyanide must be added to the enzyme in the absence of thiosulfate in order to obtain any inhibition. The same is true for sulfite (Table 4 e), and thus no inhibition is obtained in the test system by the sulfite formed during the reaction, as thiosulfate is present in the substrate. As thiosulfate in solution contains sulfhydryl groups, its protecting properties may be due to these, as cysteine and thiourea protect in the same way.

Table 4 a. Effect of metal enzymes inhibitors.

Inhibitor	Concentration * *M*	Inhibition %
Sulfide	0.1	0
Cysteine	0.1	4
Dithiodiethylcarbaminate	0.1	1
Azide	0.05	6
Cyanide	0.005	100
»	0.0005	24
Acetonitrile	0.05	6
Cyanate	0.05	0
Fluoride	0.1	0
Pyrophosphate	0.1	5
Taurocholate	0.01	53
»	0.001	26

* The indicated concentration in Tables 4 a, 4 b and 4 c refers to the incubation, the inhibitor was diluted 5-fold in the test system.

Table 4 b. Effect of carbonyl group reagents.

Reagent	Concentration *M*	Inhibition %
Cyanide	0.005	100
Cyanide + 0.05 *M* cysteine	0.005	0
Cyanide + 0.05 *M* thiourea	0.005	6
Sulfite	0.0005	99
Sulfite + 0.005 *M* thiosulfate	0.0005	0
Sulfite + 0.05 *M* cysteine	0.005	20
Sulfite + 0.005 *M* thiourea	0.0005	42
Hydroxylamine	0.0005	96
Hydroxylamine + 0.05 *M* cysteine	0.0005	6
Phenylhydrazine	0.005	98
Phenylhydrazine + 0.05 *M* cysteine	0.005	0
Semicarbazide	0.05	63
Semicarbazide + 0.05 *M* cysteine	0.05	5
Thiosemicarbazide	0.05	0

Sulfhydryl reagents: The effect of different compounds, known to react with sulfhydryl groups in proteins, was then studied. Rhodanese was found to be very sensitive to small amounts of H_2O_2 and I_2, less active were $K_3Fe(CN)_6$ and alloxan. (Table 4 c.) The enzyme was also destroyed by incubation with ascorbic acid. Cystine showed no inhibition while iodoacetate, *p*-chloromercuribenzoate and iodosobenzoate gave about 50 % inhibition in 0.001 *M*

Table 4 c. Effect of sulfhydryl reagents.

Reagent	Concentration *M*	Inhibition %
H_2O_2	10^{-4}	100
»	10^{-5}	17
I_2	10^{-5}	98
»	10^{-6}	27
$K_3Fe(CN)_6$	0.001	28
Alloxan	0.01	47
Ascorbate	0.04	95
Cystine	*	0
Iodoacetate	0.001	52
Iodoacetate + 0.005 *M* cysteine	0.001	5
p-Chloromercuribenzoate	0.001	61
p-Chloromercuribenzoate + 0.005 *M* cysteine	0.001	0
Iodosobenzoate	0.001	63
»	0.0001	31
Iodosobenzoate + 0.005 *M* cysteine	0.001	0

* Saturated solution in 0.01 *M* phosphate buffer pH 7.4.

solution. The effect obtained with the latter three compounds is weak, considering the inhibition they usually give with other sulfhydryl enzymes. Hence these results do not merit the conclusion that rhodanese is a sulfhydryl enzyme. Supporting evidence is however obtained from the protecting effect of cysteine against the inhibition with these compounds, as shown in Table 4 c. This is in accordance with the conclusions arrived at by Saunders and Himwich [8] from the inhibition obtained with iodoacetate.

INTRACELLULAR DISTRIBUTION

The purification experiments had indicated that part of the enzyme was bound to insoluble matter. Differential centrifugation experiments on sucrose homogenates of guinea pig liver also show that the activity of the homogenate was equally distributed between the mitochondrial fraction and in the supernatant. Only small amounts appeared in the nuclear fraction. The results from a typical experiment are shown in Table 5.

Here an interesting fact is apparent. The sum of activity of the nuclei, mitochondria and supernatant is greater than the original homogenate. This effect was repeatedly obtained in different experiments. Similar results for a transaminase [14] have recently been described but no explanation for this effect

Table 5. Distribution of rhodanese and rhodanese activity in fractions isolated from homogenates of guinea pig liver.

Fraction	% activity of homogenate	RU/g protein
Homogenate	100	103
Nuclei	7.0	97
Mitochondria	67.5	438
Supernatant	71.5	86

The sum of activity in nuclei, mitochondria and supernatant is 146 % of the activity in the original homogenate.

was attempted. The increase in total activity during the fractionation was obtained by washing the mitochondria with sucrose as shown by the following experiment.

Guinea pig liver was homogenized and the nuclei sedimented as before (without any washing) and their activity and the activity in the supernatant (S_1) was determined. The sum of these two activities was now found to be equal to the activity in the original homogenate. The mitochondria were then sedimented from S_1 and washed twice as before, and their activity (M) and the activity in the supernatant (S_2) was assayed. The sum of the activity in M and S_2 was now found to be about 130 % of that in S_1.

It was also found that the rhodanese activity for a cell free sucrose homogenate was only 30—50 % of that obtained in a water homogenate of the same liver, even though sucrose did not inhibit rhodanese. The rhodanese activity in homogenates with most of the mitochondria present in an intact form is thus lower than that obtained after disrupting the mitochondria. Saunders and Himwich [15] have shown that the rhodanese activity in intact cells is only a small fraction of that obtained after disintegration of the cells. They explained this as due to the small permeability of the cell membrane to thiosulfate ions. It therefore seems reasonable to ascribe the low rhodanese activity in intact mitochondria to permeability effects. It has in fact been claimed that the mitochondria are surrounded by a membrane [16–18] but this has recently been denied by Harman [19]. In any case the complex structure of the mitochondria makes the presence of permeability effects possible.

From the preceeding discussion it can be seen that our data for the intracellular distribution of rhodanese has only a relative significance, as a large part of the total activity in liver is not accounted for. It is presumeably localized in the mitochondria. It is clearly established from the high specific activity of the mitochondria as compared with the activity of the homogenate

that a large part of the rhodanese is associated with these entities (see Table 4). Since recent work by Harman [6] and Still and Kaplan [20] has shown that enzymes are transferred from the mitochondria to the supernatant during homogenization, the validity of data attained from such distribution studies is doubtful. As a consequence we have not carried out these investigations further.

SUMMARY

Rhodanese has been partially purified and some of its properties have been studied. It is inhibited by cyanide but not by the other metal enzyme inhibitors studied. The cyanide inhibition could be prevented by cysteine. Studies with inhibitors suggested that rhodanese is a sulfhydryl enzyme, but no evidence was obtained for the presence of an active carbonyl group. The intracellular distribution of rhodanese has been studied in sucrose homogenates and the enzyme found to be mainly associated with the mitochondria.

The author wishes to express his sincere gratitude to Professor Hugo Theorell and Dr Karl-Gustav Paul for kind advice and criticism in connection with the work.

ADDENDUM

Since this paper was submitted for publication we have noticed the appearance of a paper by Ludewig and Chanutin [21]. Studying the distribution of different enzymes in isotonic sucrose homogenates of rat liver, they found the main part of rhodanese activity in the mitochondria.

REFERENCES

1. Lang, K. *Biochem. Z.* **259** (1933) 243.
2. Cosby, E. L., and Sumner, J. B. *Arch. Biochem.* **7** (1945) 457.
3. Bücher, T. *Biochem. et Biophys. Acta* **1** (1947) 292.
4. Hogeboom, G. H., Schneider, W. C., and Pallade, J. *J. Biol. Chem.* **172** (1948) 619.
5. Kennedy, E. P. and Lehninger, A. L. *J. Biol. Chem.* **179** (1949) 957.
6. Harman, J. W. *Exptl. Cell Res.* **1** (1950) 382.
7. Lang, K. *Z. Vitamin. Hormon. Fermentforsch.* **2** (1949) 288.
8. Saunders, J. P., and Himwich, W. A. *Am. J. Physiol.* **163** (1950) 404.
9. Bénard, H., Gajdos, A., and Gajdos-Torok, M. *Presse Méd.* **56** (1948) 269.
10. Anson, M. L. *J. Gen. Physiol.* **23** (1939/40) 239.
11. Bergman, M., Fruton, J. S., and Fraenkel-Conrat, H. *J. Biol. Chem.* **119** (1937) 35.
12. Walti, A. *J. Am. Chem. Soc.* **60** (1938) 493.
13. Mauthner, J. *Z. Physiol. Chem.* **78** (1912) 28.

14. Müller, A. F., and Leuthardt, F. *Helv. Chim. Acta* 33 (1950) 268.
15. Saunders, J. P., and Himwich, W. A. *Am. J. Physiol.* 153 (1948) 348.
16. Claude, A., and Fullam, E. F. *J. Exptl. Med.* 81 (1945) 51.
17. Dalton, A. H., Kahler, H., Kelly, M. G., Lloyd, B. J., and Stielrich, M. *J. Nat. Cancer Inst.* 9 (1949) 439.
18. Zollinger, H. *Experientia* 4 (1948) 312.
19. Harman, J. W. *Exptl. Cell Res.* 1 (1950) 394.
20. Still, J. L., and Kaplan, E. H. *Exptl. Cell Res.* 1 (1950) 403.
21. Ludewig, S., and Chanutin, A. *Arch. Biochem.* 29 (1950) 441.

Received February 17, 1951.