RHODANESE-IRON PROTEIN ASSOCIATION IN BOVINE LIVER EXTRACTS

Marguerite Volini, Deborah Craven and Kathleen Ogata

Department of Biochemistry and Biophysics
University of Hawaii, Honolulu, Hawaii 96822

Received October 4,1977

SUMMARY: In semi-crude liver extracts, rhodanese is associated with iron protein. A component purified from mitochondrial extracts exhibits the properties of a rhodanese-apoprotein complex. It can be converted to a rhodanese-iron protein complex by incubation with iron ions, mercaptoethanol, and the sulfur-donor substrate, thiosulfate ion. The protein complex evidences an extinction coefficient close to 5000 per mole of bound iron near 400 nm. Estimates of labile sulfide for these preparations range from 0.6-0.9 mole per mole of bound iron. Furthermore, in the rhodanese-catalyzed thiosulfate-cyanide reaction, iron ion is an inhibitor competitive with cyanide suggesting that iron ion serves as a physiological sulfur-acceptor substrate for the enzyme.

Liver mitochondria contain the sulfurtransferase, rhodanese (E.C. 2.8.1.1), in large amounts (1,2). Among the physiological roles proposed for this enzyme, it has been suggested that rhodanese serves as sulfur donor to nonheme iron proteins (1-4). In support of this proposal, both the mammalian and plant enzymes have been shown to aid in reconstitution of ferrodoxin from apoferrodoxin, presumably by contributing essential "labile" sulfur atoms (3,4).

Since apoferrodoxin can be regenerated to active ferrodoxin under nonenzymic conditions (3,5), the question is raised as to whether rhodanese serves in a direct donor capacity or only to generate the necessary sulfide ions from its substrate, thiosulfate ion. The present studies of rhodanese-associated proteins from semi-crude and mitochondrial liver extracts support the former view that the enzyme is involved in the activation of nonheme iron proteins.

METHODS

Preparation of Bovine Liver Extracts. The method of Horowitz and De Toma was used for the preparation of crystalline rhodanese (6). The semi-crude extracts were supernatants from the final crystallization step which would not yield crystals after repeated treatment with ammonium sulfate solutions (7). Mitochondrial suspensions were prepared free of rhodanese activity (8). The latent rhodanese activity was liberated by repeated freezing and thawing of the mitochondrial suspension and dilution with an equal volume of 0.1 M glycine acetate buffer, pH 5.0. The suspension was centrifuged at 13,000g and the

ISSN 0006-291X

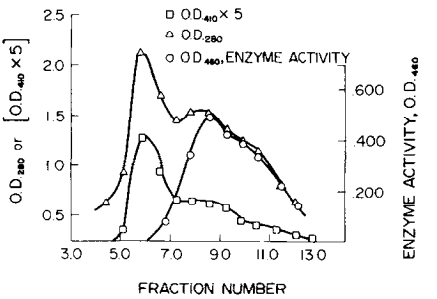

Fig. 1. Resolution of semi-crude liver extract on Sephadex G-100. The extract was prepared as described in the text. It contained 30% rhodanese and 0.1 μmole of iron/O.D. 410 nm.

pellet reextracted with cold deionized water. After centrifugation at 13,000g, the combined supernatants were brought to 2.0 M in ammonium sulfate and adjusted to pH 4.4 with cold 1 M acetic acid. After recentrifugation, most of the rhodanese activity remained in the supernatant but was precipitated by raising the ammonium sulfate concentration to 2.5 M. The pellet obtained after final centrifugation contained about 20% rhodanese. It was suspended in 1.8 M ammonium sulfate and stored at -10°. Chromatography. Columns of Sephadex G-25 or Sephadex G-100 (40-45 cm x 0.7 cm or 46.5 cm x 2.5 cm) were preequilibrated with 0.2 M tris acetate buffer, pH 8.6. Effluent fractions were collected in tared, plastic vials. Fraction volumes (0.5-1.0 ml) were calculated assuming a specific gravity of 1.0 for the solutions. SDS-Gel Electrophoresis was carried out using the procedure of Weder and Osborn (9). Iron Content was measured using a Perkin-Elmer Model 290b atomic absorption spectrophotometer. Spectral Measurements were made on a Cary Model 15 spectrophotometer. For most samples, spectra were recorded from 600 nm to 250 nm. Protein Content was measured using the method of Zamenhof (10). Kinetic Measurements. Rhodanese activity was determined as described previously (7). Assay mixtures contained 0.5-2.5 μmoles of potassium cyanide, 5-50 μmoles of sodium thiosulfate, 1% mercaptoethanol and either H_2O or 0.18-0.36 μmoles of $FeCl_3$ in a final volume of 1.0 ml. Reaction times were 15, 30, and 45 sec in most cases. Controls, timed from the addition of cyanide, corrected for the spontaneous rate of formation of colored iron-cyanide complexes so that initial rates were obtained.

RESULTS

Semi-Crude Extracts. Purified liver preparations in which rhodanese activity accounts for 20-50% of the total protein are refractory to crystallization to varying degrees. These preparations are characterized by a spectral band near 410 nm and a relatively high iron content. When chromatographed on Sephadex G-100, these preparations are resolved into three major components of differing molecular weights, all of which display absorbance at 410 nm. A chromatogram of a typical preparation is shown in Figure 1. The fractions were monitored for enzyme activity, absorbance at 280 nm and absorbance at 410 nm. They were

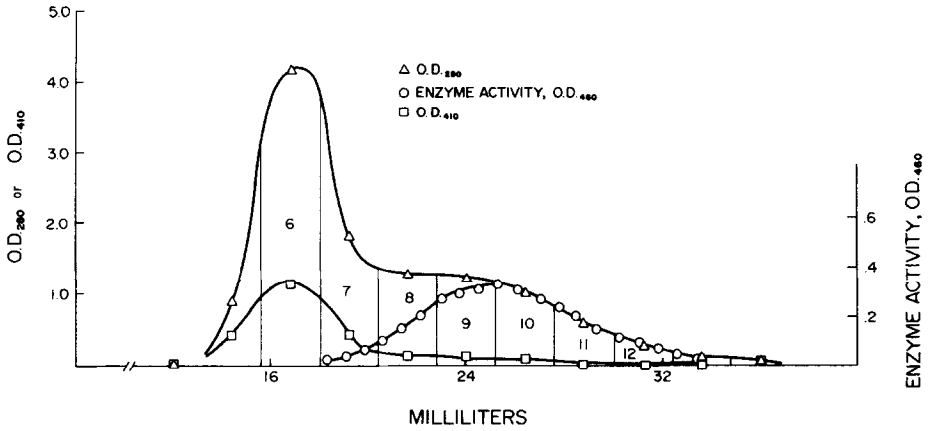

Fig. 2. Resolution of the mitochondrial extract on Sephadex G-100. The extract was prepared as described in the text. It contained 20% rhodanese.

further analyzed by sds-gel electrophoresis. Only the lower molecular weight components displayed enzyme activity (Fig. 1). However, the sds-gels showed a band characteristic of rhodanese in all fractions suggesting that the three components are aggregates of rhodanese and iron-protein which differ in their degree of polymerization.

Mitochondrial Extracts. Gel exclusion chromatography of mitochondrial preparations purified to 20% rhodanese content gave molecular weight and activity distributions similar to those observed with the semi-crude extracts (Fig. 2). Furthermore, as with the semi-crude extracts, a band characteristic of rhodanese was observed in sds-gels of all fractions, including those containing the inactive, high molecular-weight component (Fig. 3).

The iron content, however, was negligible in the low molecular-weight fractions containing enzyme activity although the rhodanese protein accounted for only 40% of the total absorbance at 280 nm.

Incorporation of Iron and "Labile" Sulfide into Mitochondrial Fractions. The ratio of absorbance to activity in fractions 9 through 12 (Fig. 2) was nearly constant. This observation suggested the presence of a rhodanese:apoprotein

TABLE I

GENERATION OF [RHODANESE:FE-PROTEIN] FROM [RHODANESE:APOPROTEIN]

Sephadex G-100 fractions from the mitochondrial extract were incubated overnight with 0.45 mM Fe ion, 25 mM $SSO_3^=$, and 0.14 M mercaptoethanol at pH 8.5. Individual fractions were rechromatographed on G-25 to remove excess iron.

Fraction No.*	Rhodanese** Content, %	$\frac{\text{Activity}}{\text{ml}}$, O.D.$_{460}$ x 10	Fe Content, μM	O.D.$_{410}$
9_{25}	35	1.10	15	0.110
10_{25}	42	0.74	9.5	0.073
11_{25}	43	0.49	7.5	0.040
12_{25}	40	0.18	3	0.015
Crystalline Rhodanese	100	1.10-3.20	0	0.000

*9_{25}, etc. denotes fraction from Sephadex G-100 column rechromatographed on Sephadex G-25 following incubation.

** $\left[\frac{\text{O.D.}_{280}\ \text{Rhodanese}}{\text{O.D.}_{280}\ \text{Total}}\right] \times 100$

complex. To test this possibility, an aliquot of each of these fractions was incubated with iron ions, mercaptoethanol and thiosulfate ions and rechromatographed on Sephadex G-25 to remove nonprotein-bound iron.

The rechromatographed fractions exhibited the brown color characteristic of nonheme iron proteins and an increased absorbance in the visible region. Their iron contents were proportional to protein concentration. Crystalline enzyme treated identically did not bind iron ions nor exhibit absorbance near 410 nm.

Before incubation, the fractions exhibited low absorbance values in the visible region contributed by a small amount of heme protein ($\leq$ 5% of the total protein). In later experiments, an aliquot of the fraction treated with mercaptoethanol and thiosulfate ions but no iron ions was rechromatographed to

Fig. 3. SDS-gels of fractions from the chromatogram described in Figure 2. Crystalline rhodanese is included at right.

serve as a measure of the residual absorbance due to the small amount of heme protein. This procedure allowed estimation of the extinction coefficient at the maxima between 400 and 430 nm as close to 5000 per mole of bound iron.

The fractions in Table I were treated with 0.14 M mercaptoethanol. Increasing the mercaptoethanol concentration to 0.19 M increased the amount of bound iron. An absorption spectrum representative of these fractions is shown in Figure 4. In this case the contribution to the visible absorbance from heme protein is negligible.

Labile sulfide was estimated in the incubated samples after chromatography on Sephadex G-25 by the method of Chen and Mortenson (11). The molar ratio of sulfide ion to bound iron ion ranged from 0.6-0.9. These values were obtained on samples which contained small amounts of rhodanese where the contribution from enzyme-bound sulfur would be negligible.

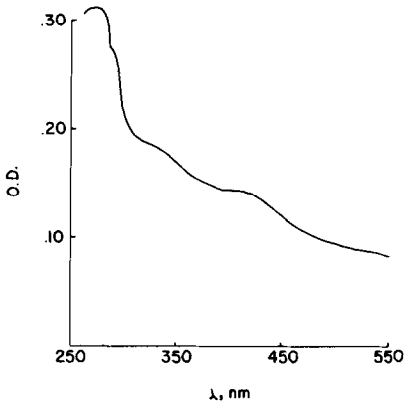

Fig. 4. Absorption spectrum of iron-protein generated by incubation of a column fraction equivalent to no. 12 in Figure 2. The incubation mixture contained 0.45 mM Fe ion, 25 mM $SSO_3^=$ and 0.19 M mercaptoethanol. It was rechromatographed on Sephadex G-25 to remove excess iron.

Iron Ion Inhibition of Thiosulfate-Cyanide Activity. The sulfur-acceptor substrate used for routine activity measurements of rhodanese is cyanide ion (1,2). Iron ions, when included in reaction mixtures containing mercaptoethanol and the donor and acceptor substrates, thiosulfate and cyanide ions, inhibit the activity of the crystalline enzyme. Iron ion, in contrast to other transition metal ions tested, is an inhibitor competitive with the acceptor substrate, cyanide ion, but uncompetitive with the donor substrate, thiosulfate ion. These results suggest that iron ion bound to protein may serve as an acceptor for the enzyme-bound sulfur atom.

DISCUSSION

Rhodanese is present solely in the mitochondria (12,13) and according to Koj is localized in the matrix (8). The present studies lend support to the view that, in the mitochondria, rhodanese functions as sulfur donor to nonheme iron protein. Mitochondrial protein extracted with rhodanese can be converted to nonheme iron protein containing labile sulfide. Furthermore, from kinetic measurements, it appears likely that iron ion can serve as sulfur-acceptor

substrate for the enzyme. In semi-crude extracts, crystallization of the enzyme may be inhibited because of binding to iron protein.

Suzuki and Kimura (14) and Lovenberg and McCarthy (15) have demonstrated the conversion of bovine serum albumin to an "artificial" nonheme iron protein by incubation with iron ions, mercaptoethanol and sulfide ions. Recently, it has been shown by Bonomi, et al (16) that the iron-sulfur flavoprotein, succinate dehydrogenase, can be reactivated by rhodanese-mediated sulfur transfer. Under the same conditions, rhodanese-mediated transfer of sulfur to bovine serum albumin was negligible. The latter observations suggest a degree of specificity for the rhodanese-mitochondrial protein interaction reported here.

ACKNOWLEDGEMENTS: This work was supported by Research Grant BMS 75-23299 from the National Science Foundation and the Office of Research Administration, University of Hawaii.

REFERENCES

1. Westley, J. (1973) in Adv. in Enzymol. (ed. A. Meister) 327-368.
2. Sörbo, B. (1975) in Metabolic Pathways (ed. D.M. Greenberg) Vol. VII, 433-456.
3. Finazzi-Agró, A., Cannella, C., Graziani, M.T. and Cavallini, D. (1971) FEBS Letters 16, 172-174.
4. Tomati, U., Matarese, R. and Federici, G. (1973) Phytochemistry 13, 1703-1706.
5. Brodrick, J.W. and Rabinowitz, J.C. (1977) in Iron-Sulfur Proteins (ed. W. Lovenberg) Vol. III, 101-119.
6. Horowitz, P. and De Toma, F. (1970) J. Biol. Chem. 245, 984-985.
7. Wang, S.-F. and Volini, M. (1973) J. Biol. Chem. 248, 7376-7385.
8. Koj, A., Frendo, J. and Wojtczak, L. (1975) FEBS Letters 57, 42-46.
9. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4405-4412.
10. Zamenhof, S. (1955) in Methods in Enzymology (eds. S.P. Colowick and N.O. Kaplan) Vol. 3, p. 702, Academic Press, New York.
11. Chen, J.-S. and Mortenson, L.E. (1977) Anal. Biochem. 79, 157-165.
12. Sorbo, B. (1951) Acta Chem. Scand. 5, 724-729.
13. De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604-617.
14. Suzuki, K. and Kimura, T. (1967) Biochem. Biophys. Res. Commun. 28, 514-519.
15. Lovenberg, W. and McCarthy, K. (1968) Biochem. Biophys. Res. Commun. 30, 453-458.
16. Bonomi, F., Pagani, S., Cerletti, P. and Cannella, C. (1977) Eur. J. Biochem. 72, 17-24.