Mitochondrial Rhodanese: Membrane-bound and Complexed Activity*

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We have proposed that phosphorylated and dephosphorylated forms of the mitochondrial sulfurtransferase, rhodanese, function as converter enzymes that interact with membrane-bound iron-sulfur centers of the electron transport chain to modulate the rate of mitochondrial respiration (Ogata, K., Dai, X., and Volini, M. (1989) J. Biol. Chem. 264, 2718–2725). In the present studies, we have explored some structural aspects of the mitochondrial rhodanese system. By sequential extraction of lysed mitochondria with phosphate buffer and phosphate buffer containing 20 mM cholate, we have shown that 30% of the rhodanese activity of bovine liver is membrane-bound. Resolution of cholate extracts on Sephadex G-100 indicates that part of the bound rhodanese is complexed with other mitochondrial proteins. Tests with the complex show that it forms iron-sulfur centers when incubated with the rhodanese sulfur-donor substrate thiosulfate, iron ions, and a reducing agent. Experiments on the rhodanese activity of rat liver mitochondria give similar results. Taken together, the findings indicate that liver rhodanese is in part bound to the mitochondrial membrane as a component of a multiprotein complex that forms iron-sulfur centers. The findings are consistent with the role we propose for rhodanese in the modulation of mitochondrial respiratory activity.

The sulfurtransferase rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) occurs widely in nature (1, 2). In heart and liver tissue, the enzyme activity is localized in the mitochondria (1–6). The activity is identified in a reaction in which cyanide is converted to the less toxic thiocyanate:

\[ \text{SSO}_3^- + \text{CN}^- \xrightarrow{\text{rhodanese}} \text{SCN}^- + \text{SO}_3^- \]

However, several studies support the role of the enzyme in the formation of 2S-2Fe and 4S-4Fe iron-sulfur centers (7–12). Related studies have shown that the sulfur transferred by rhodanese activates the electron transport proteins, succinate dehydrogenase (13) and NADH dehydrogenase (14), as well as other enzymes of oxidative metabolism, for example xanthine oxidase (15) and NADH nitrate reductase (16). With xanthine oxidase, both activating and deactivating effects are attributed to the presence of rhodanese (15). All of the effects probably arise because of rhodanese interactions with iron-sulfur centers.

Recent work done in this laboratory indicates that bovine mitochondrial rhodanese is a phosphoprotein and has led to the proposal of a bicyclic cascade system that can modulate the rate of respiration (17). In the cascade system, it is proposed that phospho- and dephosphorhodanese interact with membrane-bound components of the electron transport chain to reversibly sulfurate iron-sulfur centers. In the present studies, we have tested for membrane-bound rhodanese activity. We have shown quantitatively that in both bovine liver and rat liver mitochondria rhodanese is partitioned between membrane-bound and soluble forms, and we have obtained evidence that part of the rhodanese is associated with other mitochondrial proteins in a high molecular weight, membrane-bound complex that forms iron-sulfur centers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trisma base, sodium cholate, N,N,N-dimethyl-p-phenylenediamine-HCI, and diithiothreitol were obtained from Sigma. Sephadex gels were from Pharmacia LKB Biotechnology Inc. Chemicals for gel electrophoresis were from Bio-Rad. All chemicals were of reagent grade.

**Preparation of Mitochondria**—Four preparations of bovine liver mitochondria were used in these experiments. The isolation procedures were as described previously (17) with the following modifications for preparation C of Table I. The liver was homogenized with 0.25 M rather than 0.32 M sucrose and the mitochondrial pellet was washed with an equal volume rather than a 10-fold volume of 0.25 M sucrose. The limited washing is responsible for the lower specific rhodanese activity of preparation C relative to the other three preparations (Table I). Mitochondria were also prepared from the livers of Long Evans rats. 700 g of liver were obtained from 56 animals which ranged in weight from 300 to 350 g. The isolation procedures were as described for bovine mitochondria (17).

**Rhodanese Preparation, Activity Measurement, and Kinetic Analyses**—Pure crystalline rhodanese was prepared from bovine liver by the method of Horowiz and DeToma (18) with some modification (1/4). Rhodanese activity was measured by following the rate of thiocyanate formation. The standard assay mixtures contained 50 mM sodium thiosulfate, 50 mM potassium cyanide, 40 mM potassium dihydrogen phosphate, and 1–100 \( \mu \)l of enzyme solution in a final volume of 1.0 ml at pH 8.6. For the assay of large aliquots of diluteenzyme in buffered solutions, KHPO\(_4\) was replaced in the reaction mixtures with Tras acetate buffer, pH 8.6, 0.1 M. The reaction was initiated by the addition of enzyme and stopped by the addition of 0.5 ml of 15% formaldehyde. 1.5 ml of ferric nitrate reagent, containing 100 g of Fe(NO\(_3\))\(_3\)-9H\(_2\)O and 200 ml of 65% HNO\(_3\) (1500 ml) was added, yielding the characteristic red-brown color of the acidic iron-thiocyanate complex. The intensity of the color was measured at 460 nm in a Bausch & Lomb Spectronic 20. For crude extracts, turbidity was removed by centrifugation before the absorbance was measured. Measurements were corrected for spontaneous rates by omission of enzyme from the assay mixture and for color contributed by crude extracts, by omission of cyanide from the assay mixture. Rates were measured at several intervals, usually 15 s, 30 s, and 1 min to ensure initial velocities were observed. Most samples were assayed in the presence and absence of 0.14 M \( \beta \)-mercaptoethanol. Although sulfhydryl reagents in the presence of oxygen are known inhibitors of sulfur-free rhodanese (19), when added in the presence of thiosulfate, they prevent the inactivation of dilute enzyme which results from autoxidation. For kinetic analyses, rare measurements of thiocyanate formation were carried out under conditions similar to those used for the standard assay of enzyme activity. The reaction

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tracts were chromatographed at 4°C on Sephadex G-100 and less rhodanese activity from free or dissociated rhodanese activity, ex-

and 0.7 is the liquid fraction of the pellet.

volumes of 0.3-0.7 ml were added to columns 46-49

experiments, the protein in the extracts was concentrated by precip-

0.5-1.0 ml, were calculated from weights using a specific gravity of

of the supernatant, the pellet, now much reduced in size, was re-

extracted with buffer containing cholate to ensure complete recovery

of membrane-bound activity.

Calculation of Extract Recoueries—The activities recovered in the extract supernatants were calculated from the formula:

\[ A_i = a_i V \]

where \( a_i \) is the activity per ml and \( V \) is the volume in ml. The amount of soluble activity expected for a given supernatant after the first extraction step (Table I) was estimated from the formula:

\[ S_i = S_i' \frac{X_i}{X_i'} \]

where \( S_i \) is the activity measured in the first extract supernatant, \( X_i \), the fraction of the total soluble activity obtained at a given step, was estimated from the following expressions:

1) for supernatants prior to the addition of cholate,

\[ X_i = \left(1 - \sum_{j=0}^{i-1} \frac{D_j}{(D_j + 0.7)} \right) \]

where \( D_j \) represents the parts of buffer added to one part of pellet and 0.7 is the liquid fraction of the pellet.

2) Upon addition of cholate, the pellet is reduced from 1.0 to 0.4 parts of which 0.7 is the liquid fraction,

\[ X_i = \left(1 - \sum_{j=0}^{i-1} \frac{D_j + 0.6}{D_j + 0.9} \right) \]

The activity remaining at the final cholate step is given by,

\[ A_{i-1} = \frac{S_i}{X_i} \left(1 - \sum_{j=0}^{i-1} X_i \right) \]

Molecular Exclusion Chromatography—For resolution of complexed rhodanese activity from free or dissociated rhodanese activity, extracts were chromatographed at 4°C on Sephadex G-100 and less often on Sephadex G-200. Void volumes of the columns were esti-
mated from the elution volume of blue dextran. For routine deter-
minations of the amount of complexed rhodanese activity, extract volumes of 0.3-0.7 ml were added to columns 46-49 v 0.7 cm; effluent fractions were collected in tared plastic vials and fraction volumes, 0.5-1.0 ml, were calculated from absorbance readings using a specific gravity of 1.0. For resolution of the large amounts of complexed needed for kinetic analyses and for studies on iron and sulfur uptake, extract volumes of 2-5 ml were chromatographed on columns 50 x 1 cm or 50 x 2.5 cm, effluent fractions of 2-5 ml were collected. In some of these experiments, the protein in the extracts was concentrated by precip-

itation with ammonium sulfate, final concentration 2.5 M, prior to chroma-
tography. The Sephadex columns were equilibrated and eluted with Na-K phosphate buffer, 0.2 I, pH 7.1, or with the same buffer containing 20 mM cholate. However, when iron content was to be measured by atomic absorption, the complex was resolved in Tris acetate buffer, 0.05 I, pH 8.6, because of interference by phosphate. For protein determinations on the latter fractions, aliquots were exchanged into phosphate buffer on Sephadex G-25 because of interference by Tris with the biuret reaction (21). In some experiments, the soluble activity was extracted into 0.1 M glycine acetic acid buffer, pH 5.0. Samples of these extracts were usually resolved on columns equilibrated with phosphate buffer.

SDS-Gel Electrophoresis—The method of Weber and Osborn (22) was used with bovine serum albumin, catalase, ovalbumin, yeast alcohol dehydrogenase, crystalline rhodanese, myoglobin, cytochrome c, and lysozyme as marker proteins.

Measurements of Iron and Sulfur Uptake—1-2 ml aliquots of the pooled fractions containing complexed rhodanese, 1-20 mg, in Tris acetate buffer, 0.05 I, pH 8.6, were incubated with 0.35-0.92 mM ferric chloride, 4-43 mM sodium thiosulfate, and 0.14-0.18 mM β-mercaptoethanol usually for 16-18 h at 4°C. Excess reagents were removed by chromatography on Sephadex G-25 in the same buffer. For the time course experiments 0.4-ml aliquots of the reaction mixtures, total volumes 1.24 ml including 0.35 mM ferric chloride, 4.0 mM thiosulfate, and 0.14 M β-mercaptoethanol, were chromatographed 30 min, 2 min, and 1 min later using atomic absorption using a Perkin-Elmer 290B spectrophotometer and absorption spectra were recorded from 600 to 200 nm on a Cary Model 15 UV-visible spectrophotometer. The content of acid-labile sulfide was measured by a modification of the procedure of Chen and Mortenson (23). The reaction mixture, 1.65 ml, contained 1.0 ml of 1% zinc acetate, 0.05 ml of 12% NaOH, up to 0.3 ml of sample, and 0.2 ml of N,N-dimethyl-p-phenylene diamine-HCl. The concentration of acid-labile sulfide was estimated using the extinction coefficient given by Chen and Mortenson (23), 3.04 × 10^5 M⁻¹ cm⁻¹.

RESULTS

Membrane-bound Rhodanese Activity—The yields of rho-
danese activity in supernatants obtained from successive ex-
tractions of four different bovine liver mitochondrial prepa-

rations are listed in Table I. In the initial extraction steps, the lysed mitochondrial suspensions were diluted with phos-
phate buffer and centrifuged for removal of soluble rhodanese activity. In the later extraction steps, the mitochondrial pel-
lets were treated with phosphate buffer containing sodium cholate to release membrane-bound rhodanese activity. Most of the soluble activity was removed in the initial extract

preparations A and B were more than 5 fold the values

of Table I. The bound activity represents more than 40% of

the soluble activity of the mitochondria and comprises about

1The abbreviation used is: SDS, sodium dodecyl sulfate.
Yields of soluble and bound rhodanese activity from bovine liver mitochondria

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Extraction Activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Soluble</td>
</tr>
<tr>
<td>Step</td>
<td>Ratio</td>
</tr>
<tr>
<td>A (1.6 g)</td>
<td>I 1:1.5</td>
</tr>
<tr>
<td></td>
<td>II 1:1</td>
</tr>
<tr>
<td></td>
<td>III 1:1</td>
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<tr>
<td></td>
<td>IV 1:2</td>
</tr>
<tr>
<td></td>
<td>V 1:1</td>
</tr>
<tr>
<td>B (3.9 g)</td>
<td>I 1:1</td>
</tr>
<tr>
<td></td>
<td>II 1:1</td>
</tr>
<tr>
<td></td>
<td>III 1:1</td>
</tr>
<tr>
<td></td>
<td>IV 1:2</td>
</tr>
<tr>
<td></td>
<td>V 1:1</td>
</tr>
<tr>
<td>C (2.5 g)</td>
<td>I 1:1.8</td>
</tr>
<tr>
<td></td>
<td>II 1:1.5</td>
</tr>
<tr>
<td></td>
<td>III 1:2</td>
</tr>
<tr>
<td></td>
<td>IV 1:1</td>
</tr>
<tr>
<td>D (0.6 g)</td>
<td>I 1:2</td>
</tr>
<tr>
<td></td>
<td>II 1:7</td>
</tr>
<tr>
<td></td>
<td>III 1:1</td>
</tr>
</tbody>
</table>

30% of the total activity. Preparation C, which was less purified as indicated by the lower specific activities observed, gave close to the same ratio of membrane-bound to soluble rhodanese as the other three preparations.

For the experiments of Table I, the soluble activity was extracted with phosphate buffer at neutral pH. In the procedure commonly employed for the isolation of crystalline bovine liver rhodanese (18), 0.1 mM glycine acetate buffer at pH 5.0 is used for tissue extraction. In Table III, the recoveries of rhodanese activity in soluble and bound fractions are compared for equal aliquots of a mitochondrial suspension subjected to initial treatment with phosphate and glycine acetate buffers. In all of the experiments, the bound activity was extracted with cholate in phosphate buffer at pH 7.1 because of the limited solubility of cholate at acidic pH.

As shown in the table, total recoveries of activity were the same for initial extraction with either buffer. Furthermore, the partitioning of activity between soluble and bound fractions was reproducible among determinations initiated with the same extraction buffer. However, the ratio of bound to soluble activity was reduced from an average value of 0.43 for phosphate buffer to 0.24 for glycine acetate, indicating that part of the membrane-bound rhodanese is solubilized by glycine acetate buffer at pH 5. Other membrane-bound proteins in addition to rhodanese are also solubilized by glycine acetate buffer at pH 5 relative to phosphate buffer at neutral pH. For equal aliquots of the same mitochondrial suspension, the pellet weight obtained by centrifugation following glycine acetate extraction was 0.75 of that obtained following phosphate extraction. Biuret determinations done on the pellets gave amounts of protein in similar proportion.

Complexed Rhodanese Activity—A typical elution diagram of the rhodanese activity in cholate extracts of bovine liver mitochondria chromatographed on Sephadex G-100 is shown in Fig. 1. Most of the enzyme activity was eluted at a K, value of 0.29, which corresponds to free or dissociated rhodanese. In addition, a small amount of activity was eluted at the void volume of the column, indicating that part of the membrane-bound rhodanese is present in complexed form. Complexed rhodanese activity was resolved from the extract whether chromatography was performed in the presence or absence of cholate.

The rhodanese complex was also eluted at the void volume of columns of Sephadex G-200, suggesting that it has a mass greater than 200,000 daltons. Catalase, M, = 232,000, which was used as a marker protein in these experiments was eluted...
from the same Sephadex G-200 columns at somewhat larger volumes than the rhodanese complex. The complexed rhodanese activity was stable to rechromatography at variable pH and/or ionic strength or after treatment with cholate or Triton X-100. However, complex rechromatographed in phosphate buffer containing 50 mM thiosulfate and 10 mM dithiothreitol showed limited dissociation (<5%) to free rhodanese (Fig. 2). Activity recoveries in these rechromatograms were in excess of 100%, suggesting that the specific thiocyanate-forming activity of rhodanese bound in the complex is lower than that of rhodanese free in solution. The additional activity was eluted at the $K_v$ value of free rhodanese.

SDS-gel electrophoresis indicated that in the complex rhodanese is associated with other mitochondrial proteins. Following incubation with SDS and $\beta$-mercaptoethanol, gel electrophoresis gave rise to bands corresponding to polypeptide chains of various sizes including that of the pure bovine rhodanese of $M_v = 33,000$. Furthermore, in detailed kinetic analyses using thiosulfate and cyanide ions as the variable substrates, the stable complex preparations exhibited $K_v$ values for both substrates that were virtually identical to those measured for bovine liver mitochondria. We have previously reported the presence of a stable rhodanese-protein complex in soluble extracts of bovine liver mitochondria (24). In the present work, both the soluble and cholate extracts prepared from single aliquots of lysed mitochondria were chromatographed on Sephadex G-100. From the sum of the complexed activities measured in the two chromatograms, the total complexed activity in the mitochondria was estimated as 0.25 unit/mg of protein. The same amount of complexed activity was obtained whether phosphate or glycine acetate was used as initial extraction buffer.

Dissociable Rhodanese Activity—Experiments in which the lysed mitochondrial suspensions were treated directly with cholate showed that in the complexed preparations the yield of rhodanese activity was inversely proportional to the yield of complexed rhodanese activity. The relationship can be formulated as follows:

$$F = -aC + \text{maximum activity}$$

where $F$ and $C$ are the observed activities for free and complexed rhodanese, respectively. From the slope of the plot, $a$, it is estimated that the thiocyanate-forming activity of free rhodanese is 8-fold that of the dissociable complexed rhodanese.

The minimal amount of complexed rhodanese activity observed in these experiments was 0.25 unit/mg of protein. The value was obtained at extraction ratios ~1:16 (mitochondrial weight/buffer volume). It represents stable, complexed activity and is the same as the value estimated from the sum of complexed activities measured in the separate soluble and cholate extractions (see above).

Membrane-bound and Complexed Rhodanese Activity in Rat Liver Mitochondria—2.0 g of rat liver mitochondria were initially extracted with five parts of phosphate buffer, 0.2 I, pH 7.1. After centrifugation and removal of the supernatant, the membranous pellet was resuspended in two parts of phosphate buffer, 0.4 I, pH 7.1, and sufficient cholate was added to give a final concentration of 20 mM. The yield of cholate-solubilized activity in three separate determinations ranged from 27 to 30%. Chromatography of the cholate extracts on Sephadex G-100 resolved 5.6-6.0% of the cholate-solubilized activity as the high molecular weight rhodanese complex. The fractions of membrane-bound and complexed rhodanese activities obtained with the rat liver preparation were virtually identical to those measured for bovine liver mitochondria. However, the amount of rhodanese activity measured in homogenates per g of tissue and the specific activities of mitochondrial preparations from livers of both Long Evans and Wistar rats were 1.5-2.0-fold that of bovine liver.

Formation of Iron-Sulfur Centers by the Rhodanese-Protein Complex—It has been known for many years that the reconstitution of iron-sulfur centers in clostridial and other apoferredoxins can be accomplished by chemical methods, using sulfide, iron ions, and a mercaptide reducing agent, either $\beta$-mercaptoethanol, dithiothreitol, or dihydrolipoate (8, 25-27). In their original studies, Malkin and Rabahowitz (25) did not observe reconstitution to active ferredoxin if any one of the
three components was omitted from the incubation mixture, but, in later work from the same laboratory, it was shown that only sulfide and iron ions were required to produce a functional ferredoxin, provided the sulfhydryl groups of the apoprotein were maintained in a reduced state during extrusion of the iron-sulfur cluster by treatment with trichloroacetic acid (26).

Because of the toxicity of hydrogen sulfide (28) and the lack of specificity of the chemical reconstitution process (29, 30), the rhodanese-thiosulfate system in the presence of a thiol reducing agent has been investigated as a source of sulfide for the formation of iron-sulfur centers (8–12). During the rhodanese-catalyzed generation of sulfide, the outer sulfur of thiosulfate is transferred to a dithiol or, at a lower rate, to a monothiol forming a highly reactive persulfide product which breaks down spontaneously, generating sulfide and an oxidized thiol:

$$\text{SSO}_3^- + \text{XSH} \rightarrow \text{SH}^- + \text{XS}^- + \text{XS}$$

$$\text{SSO}_3^- + \text{rhodanese} \rightarrow \text{SO}_4^{2-} + \text{or}$$

In the absence of rhodanese, the spontaneous production of sulfide in these systems, with or without added iron ions, is negligible under the conditions used for the formation of iron-sulfur centers (0–7 °C and pH 7.0–8.6).

In support of the view that rhodanese functions in the mitochondria to activate iron sulfur proteins, we have shown that in mitochondrial extracts rhodanese is associated with proteins that bind iron-sulfur centers (31). We have, further, demonstrated the time-dependent formation of iron-sulfur centers by the high molecular weight, stable rhodanese-protein complex separated from soluble extracts of bovine liver mitochondria (24). In the latter experiments, individual fractions containing the soluble complex were incubated with thiosulfate, iron ions, and &beta;-mercaptoethanol. &beta;-Mercaptoethanol, although a poor substrate for rhodanese, was selected as reductant in preference to a dithiol to limit the amount of sulfide produced by the rhodanese system, thereby minimizing possible side reactions. Aliquots of the complex fractions were chromatographed on Sephadex G-25 at increasing incubation times and tested for iron uptake, for acid-labile sulfide uptake, and for increasing absorbance near 410 nm. Identical rates were observed for stoichiometric increases in uptake and absorbance, indicating that iron sulfur centers were formed.

In the present studies we have similarly tested the stable rhodanese-protein complex that was resolved from cholate extracts of bovine liver mitochondria for iron uptake, for acid-labile sulfide uptake, and for attendant changes in absorbance. Table IV summarizes the limiting values obtained in these experiments and for comparison includes limiting values for complex resolved from soluble extracts. Mean values of 30 ± 1 and 31 ± 1 nmol/mg protein were observed for the uptake of iron and acid-labile sulfide, respectively, by the complex from cholate extracts. Uptake was proportional to the amount of protein over a range from 3.6 to 17 mg (Table IV, preparations 1–4). It can be concluded that these values approach maximal specific binding, since close to the same uptake was observed when the free iron concentration was varied 8-fold. The values for uptake by the soluble complex were close to those observed for the cholate complex, 31 ± 1 and 31 ± 3 nmol/mg protein for iron and acid-labile sulfide uptake, respectively. In all of the experiments, thiosulfate, which was the source of the bound sulfur, was present in the incubation mixtures in greater than 10-fold excess over the amount of iron added. The absorbance changes at 410 nm per mol of iron uptake were characteristic of nonheme iron-sulfur centers, ranging from 5300 to 6100 M⁻¹ cm⁻¹.

With aged preparations of the cholate complex which had lost part or all of their rhodanese activity, reduced or negligible rates of iron and acid-labile sulfide uptake were observed. The rates of uptake, however, could be increased or completely restored by the addition of pure bovine liver rhodanese at a concentration of $1 \times 10^{-7}$ M (2.5 units/ml). For example, a sample of the complex preparation listed first in Table IV, which had lost 52% of its rhodanese activity upon standing at 4 °C, exhibited iron uptake of 3.0, 8.3, and 32.4 nmol/mg at 30 min, 2 h, and 16 h, respectively. A sample of the same preparation to which pure crystalline rhodanese was added in a concentration of $1 \times 10^{-7}$ M evidenced iron uptake at the same incubation times of 6.6, 18.2, and 33.2 nmol/mg, respectively. (We have reported previously that crystalline rhodanese does not bind iron-sulfur centers (31).)

Evidence presented above shows that in reaction mixtures with thiosulfate as the source of sulfide, the rate of formation of iron-sulfur centers is negligible in the absence of rhodanese. The observations described here with aged complex preparations show further that the rate of formation of iron-sulfur centers by the complex is dependent on rhodanese concentration.

The increase in the rate of iron-sulfur uptake was not linearly dependent on thiocyanate-forming activity, when added as pure crystalline rhodanese. For the sample of aged complex described above, the amount of pure rhodanese added had thiocyanate-forming activity equivalent to 10-fold the residual thiocyanate-forming activity of the complex, but gave rise to only about a 2-fold increase in the rate of iron-sulfur center formation at short incubation times. Two factors may account for the apparent discrepancy: 1) a lower efficiency of free rhodanese in stimulating iron-sulfur center formation when compared with rhodanese bound in the complex, and 2) a lower specific thiocyanate-forming activity of rhodanese bound in the complex relative to free rhodanese (as is observed for rhodanese in the dissociable complexes). Given that the latter is the case, the actual amount of rhodanese in the stable complex that is active for formation of iron-sulfur centers may approach the amount of pure rhodanese added.

The values in Table IV were obtained after 16–18 h incubation. In other experiments with fresh complex preparations from cholate extracts, iron and sulfur uptake and absorbance

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**Table IV**

<table>
<thead>
<tr>
<th>Source of complex preparation</th>
<th>Fe (\text{nmol}^a)</th>
<th>Fe (\text{nmol}^a)</th>
<th>Fe/S</th>
<th>(\epsilon_{\text{S}}/\text{Fe}^b)</th>
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</thead>
<tbody>
<tr>
<td>Cholate extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial extraction, Na-K phosphate, pH 7.1</td>
<td>85</td>
<td>2.6</td>
<td>33</td>
<td>34</td>
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<tr>
<td>Initial extraction, glycine phosphate, pH 7.1</td>
<td>32</td>
<td>0.4</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>Soluble extract</td>
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<tr>
<td>Na-K phosphate, pH 7.1</td>
<td>130</td>
<td>3.5</td>
<td>ND</td>
<td>32</td>
</tr>
<tr>
<td>Tris acetate, pH 8.6</td>
<td>53</td>
<td>1.6</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Added (^a)</td>
<td>40</td>
<td>0.9</td>
<td>30</td>
<td>27</td>
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<tr>
<td>Free (^x)</td>
<td>50</td>
<td>1.6</td>
<td>32</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^a\) Per mg of protein.

\(^b\) At 410 nm.

\(^x\) Assuming stoichiometric uptake of Fe.

\(^d\) ND, not determined.

\(^\epsilon\) \(\epsilon_{\text{S}}/\text{Fe}\).
changes at 410 nm were measured at 30 min, 2 h, and 18 h. The results indicated the same rate of change for the parameters. The observations that iron uptake was dependent on acid-labile sulfide uptake, that the concentration ratio of iron and sulfur taken up by the complex was 1.0 (Table IV), that the increase in extinction coefficient at 410 nm was close to 6000 M⁻¹ cm⁻¹ mol⁻¹ of iron taken up, and that the rates of change in uptake and absorbance were the same indicate that iron-sulfur centers were formed. By these criteria, the results of similar experiments showed that the rhodanese complex separated from cholate extracts of rat liver mitochondria also contained iron-sulfur centers. However, the amounts of iron and sulfur taken up and the absorbance change at 410 nm were increased relative to the values observed for the bovine complex. The limiting values per mg of rat complex protein were 70 nmol, 70 nmol, and 0.40 absorbance units, respectively. The absorbance change is to be compared with the value 0.18 A/mg of protein for the bovine complex. The extinction coefficient per mol of iron for the complex from rat mitochondria was close to that of the bovine complex, 5900 M⁻¹ cm⁻¹.

**DISCUSSION**

The Intramitochondrial Distribution of Rhodanese Activity—Early studies by Sorbo (2), Ludewig and Chanutin (3), and DeDuve et al. (4) established that rhodanese activity is localized in the mitochondria of mammalian liver cells. A later study by Koj et al. (5), which employed digitonin fractionation, further restricted the rhodanese activity to rat liver mitochondria to the mitoplasts. The latter investigators measured most of the rhodanese activity in the supernatant fraction following rupture of the mitoplasts by sonication. They reported ~5% of the activity in the sedimented fraction. The low yield of membrane-bound activity observed in their experiment may be a consequence of a number of factors, for example failure to extract the sedimented fraction with a membrane-solubilizing agent, incomplete sedimentation of inner membrane particles, or inactivation of the bound rhodanese by autodigestion (see below). Both of the latter phenomena could result from adverse conditions of sonication. In the published report of the work, the conditions of sonication were not described.

The yield of membrane-bound rhodanese activity obtained in the present studies from four different preparations of bovine liver mitochondria and three separate determinations done on rat liver mitochondria was more than 40% of the amount extracted in the soluble fraction and about 30% of the total activity. That these values reflect rhodanese binding at high affinity sites on the mitochondrial membrane is indicated by the fact that the fraction of bound enzyme was virtually unchanged by the number of extractions done in the absence of cholate (Table I). The values are minimal estimates of the fraction of rhodanese bound under physiological conditions since enzyme which dissociated from low affinity sites upon lysis in suspending medium could not be distinguished from free rhodanese by the procedures used.

In a previous study (17), we have reported that phosphorylated rhodanese, when free of substrate sulfur, undergoes autodigestion, forming insoluble aggregates. It can be concluded that the oxidized phosphoenzyme does not account for a significant part of the rhodanese activity measured in the membranous pellet because the oxidized phosphoenzyme is inert to thiocyanate formation. By way of contrast, the rhodanese bound in the membranous pellet exhibits thiocyanate-forming activity.

**Rhodanese Interactions in the Stable Complexes and an Estimate of the Fraction of Rhodanese in Complexed Form—**

It was observed in this study that the stable rhodanese-protein complexes were resolved by gel electrophoresis following incubation with SDS in the presence of β-mercaptoethanol but not by chromatography with surfactant-containing buffers in the absence of a thiol reducing agent. It is inferred from these observations that in the stable complexes rhodanese is associated with other mitochondrial proteins through disulfide bonds. As yet, it has only been possible to dissociate a small amount of rhodanese which is active in catalyzing thiocyanate formation through treatment of the stable complex preparations with the reducing agent, dithiothreitol. In view of the complexity of rhodanese-sulfur interactions, this is not surprising. As ordinarily isolated, the crystalline enzyme contains four free sulfhydryl groups, one of which is essential for thiocyanate-forming activity. The molecule is susceptible to oxidation (17, 19, 32–35), especially in the phosphorylated form, and has the potential to undergo disulfide interchange interactions.

The minimal yield of complexed rhodanese activity measured either by direct extraction of lyed bovine mitochondria with buffer containing cholate (Fig. 3) or by separate extractions of the mitochondria with buffer and buffer containing cholate was 0.25 unit/mg of suspension protein. The value represents stable, complex rhodanese activity. By direct extraction with small volumes of buffer containing cholate, the yield of complexed rhodanese activity was increased 1.7-fold (Fig. 3), the additional activity arising from rhodanese which is dissociated at higher dilution. The additional complexed activity can be assigned to the membrane-bound enzyme fraction since the yield of complexed activity from supernatants obtained by minimal dilution of lyed mitochondrial in the absence of cholate was unchanged from the value routinely observed in the soluble fraction prepared at higher extraction volumes.

The specific activity of the dissociable rhodanese when bound in the complex is estimated as one-eighth that of free rhodanese. Correcting for the lower specific activity, the relative amounts of complexed to free rhodanese in the mitochondria is 0.25; assuming a similar specific activity for rhodanese bound in the stable complexes, the ratio is increased to 0.53. This calculation implies that the amount of rhodanese in complexed form is more than one-third of the total enzyme present in the mitochondria. The fraction of rhodanese that is complexed in situ may be even higher than 0.53 because the maximum amount of dissociable complex that can be observed is limited by the dilution used in the preparation of the mitochondrial suspension and by the further dilution incurred upon chromatography.

**Rhodanese and the Formation of Iron-Sulfur Centers—**

It has been reported by two laboratories that bovine serum albumin can be converted to an artificial iron-sulfur protein by incubation with sulfide and iron ions under conditions which generate free sulfhydryl groups (29, 30). The lack of specificity of the chemical reconstitution process together with the toxicity of hydrogen sulfide has provoked interest in enzyme systems that can produce the necessary sulfide for the reconstitution process under controlled conditions by conversion of nontoxic substrates. The reduction of sulfide to sulfide does not occur in animal tissues (30), but there are three sulfurtransferases known which, in the presence of their substrates and an thiol reducing agent, can convert divalent sulfur to sulfide. These enzymes are rhodanese, β-mercapto-pyrurate sulfurtransferase, and thiocarbonate reductase (37). It is probable that all three enzymes generate sulfide through spontaneous breakdown of reactive persulfide products (37).

Of these enzymes, rhodanese is the best-characterized. It has been studied extensively in the formation of iron-sulfur
centers (7-16, 24, 31, 38, 39). Using the rhodanese-thiosulfate system and a source of iron ions, apoferrredoxin from spinach, parsley, cabbage, and Clostridia have been successfully reconstituted (8-12). In the studies, bovine liver rhodanese was used to reactivate plant and bacterial ferredoxins. Nevertheless, some evidence for the specificity of the rhodanese-apoprotein interactions was obtained. For example, in studies with spinach ferredoxin (8, 9) it was reported that the rhodanese-thiosulfate system was more efficient in both the rate and extent of reconstitution compared with the chemical process, although the maximum sulfide concentration produced by the rhodanese system was at most 10% of the sodium sulfide added. The rate of chemical reconstitution was independent of the sulfide concentration over a range from 0.2 to 1.36 mM, whereas the rate was linearly dependent on the rhodanese concentration up to a concentration of 1 × 10^{-5} M. Furthermore, it was observed that the holoferreredoxin was an inhibitor of the rhodanese-catalyzed production of sulfide and that reconstitution took place, in the absence of a thiol, at the expense of sulphydryl oxidation in rhodanese, suggesting a close interaction between the two proteins.

Of direct relevance to the present studies, it has been shown that the rhodanese-thiosulfate system can restore activity to both succinate dehydrogenase (13, 39) and NADH dehydrogenase (14), following losses in chemical and functional properties as a result of altered iron-sulfur centers. In the work on succinate dehydrogenase, direct transfer of substrate sulfur from rhodanese to the dehydrogenase was demonstrated. In the same experiments, rhodanese did not donate sulfur to bovine serum albumin, indicating that the enzyme system exhibits a degree of specificity for the acceptor protein not observed with the chemical reconstitution system.

The Physiological Role of Membrane-bound and Complexed Rhodanese—The work from other laboratories described in the preceding section shows that bovine liver rhodanese in the fully reduced state with four free sulphydryl groups can transfer sulfane sulfur (divalent sulfur bonded only to sulfur) (7-16, 24, 31, 38, 39). Using the rhodanese-thiosulfate system and a source of iron ions, apoferredoxins from spinach, parsley, cabbage, and Clostridia have been successfully reconstituted (8-12). In the studies, bovine liver rhodanese was used to reconstitute plant and bacterial ferredoxins. Neverthe-

Inhibitory and stimulatory effects of these systems are already known. The phosphorylation/dephosphorylation of rhodanese and the sulfuration/desulfuration of iron-sulfur centers by rhodanese and phosphorhodanese can be viewed as a bicyclic cascade system (17). In the mitochondria, the system has the potential to modulate the rate of respiration (17). The rhodanese cycle, we propose, is the terminal phase of pathways by which hormones and neurotransmitters signal changes in oxygen consumption and ATP production. In our working model of the cascade system, we have proposed that rhodanese interacts directly with membrane-bound components of the respiratory chain and that rhodanese reconstitutes iron-sulfur centers in situ. The results of the present studies are in accordance with these proposals. They show that liver rhodanese is bound to the mitochondrial membrane as a component of multiprotein complexes that form iron-sulfur centers using the endogenous rhodanese activity.

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