Some Molecular Properties of Rhodanese*

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SUMMARY

Fluorescence studies of crystalline bovine liver rhodanese indicate that the reactive sulfhydryl group is in a region which can participate in hydrophobic (apolar) interactions. Fluorescence polarization studies confirm that rhodanese exists in a mobile monomer-dimer equilibrium. Experiments with hydrophobic probing reagents indicate that the appearance of hydrophobic regions is correlated with the dissociation of dimers to monomers.

Rhodanese (thiosulfate: cyanide sulfurtransferase, EC 2.8.1.1) catalyzes the transfer of the planetary sulfur atom from thiosulfate to any of a number of nucleophilic acceptors, including cysteine and dihydrolipoate (2). Rhodanese with a molecular weight of 37,000 (3, 4) has been shown to be a dimer in equilibrium with identical monomers by the methods of ultracentrifugation, gel filtration, and peptide mapping analysis (5). The appearance of a stable dimer in rhodanese preparations exposed to air, as well as pH activity data, led to the suggestion that repulsion of sulfhydryl groups lying close together in the reversible dimer causes dissociation to monomers by ionic repulsion (5, 6). The more reactive sulfhydryl group of the two present in the monomer, was shown to be essential for the enzymic activity by chemical modification studies using sulfhydryl-directed reagents (6). A variety of approaches led to the conclusion that one of the 4 tryptophanyl residues of the rhodanese monomer is at the active site and is in close proximity to the implicated sulfhydryl group (6-8). The work reported here indicates that the active site sulfhydryl group behaves as if it were in a region which can participate in apolar interactions. The evidence suggests that interactions involving this hydrophobic region are also important in maintaining the dimeric structure of rhodanese.

EXPERIMENTAL PROCEDURE

Rhodanese—Crystalline bovine liver rhodanese was prepared by the method of Horowitz and DeToma (9) and stored frozen as a crystal suspension in 1.0 M ammonium sulfate. Enzyme activity was measured by the rate of thiocyanate formation from thiosulfate and cyanide as previously described (6). Protein concentrations were determined either spectrophotometrically at 280 nm, with an absorbance coefficient of 1.75 cm⁻¹ per mg (3), or by a modified biuret method (10), with crystalline rhodanese as a standard. Unless otherwise indicated, the free sulfhydryl groups of rhodanese were determined according to the method of Ellman (11) as modified by Wang and Volini (6).

Small molecules were removed from enzyme solutions by use of a column (2.5 × 60 cm) of Sephadex G-25 (medium grade). Ultraviolet spectra were determined with a Cary model 15 recording spectrophotometer. All readings were taken using 1.5-ml quartz cuvettes having a 1-cm path length.

Enzyme Modification—The methods of Wang and Volini were used to prepare rhodanese inactivated with m-dinitrobenzene, β-mercaptoethanol, or iodoacetate (6). These inactivation procedures involve reactions of the enzymic sulfhydryl groups.

2-p-Toluidinyl napthalene-6-sulfonate was synthesized by the method of McClure and Edelman (12). The correspondence of this product to that previously reported was shown by the identity of the fluorescence emission and ultraviolet absorption spectra. Two methods were used for the fluorescent labeling of rhodanese. (a) Ten milligrams of enzyme were added to 1.0 ml of phosphate buffer, pH 7.4, at an ionic strength of 0.2 and 0°. To this, 50 μl of a 10⁻⁴ M solution of either 1-dimethylaminonaphthalene-6-sulfonyl chloride or fluorescein isothiocyanate in acetone were added with stirring, and the resulting suspension was stirred overnight in the cold. (b) Ten milligrams of Celite onto which had been adsorbed 1 mg of either DNS¹ or fluorescein isothiocyanate were added to 10 mg of enzyme in phosphate buffer, pH 7.4, with stirring at 0°. These were stirred for ½ hour in the case of DNS-Celite or for 4 min in the case of fluorescein isothiocyanate—Celite.

After reaction by either of the above methods, the suspensions were centrifuged in the cold. The supernatant solutions were placed immediately on a Sephadex G-25 column and eluted with glycine buffer, pH 9.4. Under ultraviolet illumination, the column showed a large separation between the zones corresponding to labeled enzyme and free dye. The effluent containing the enzyme was collected in several fractions, all of which had the same ratio of protein absorbance to dye absorbance. The conjugate with the greatest enzyme activity was obtained by the use of fluorescein isothiocyanate in Method b.

1 The abbreviations used are: DNS, 1-dimethylaminonaphthalene-6-sulfonyl chloride; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; FMA, fluorescein mercuric acetate; TNS, 2-p-toluidinyl napthalene-6-sulfonate.

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Fluorescence Measurements—All fluorescence measurements were made with an Amino-Bowman spectrophotofluorometer fitted with a high intensity xenon continuum light source and a liquid-cooled cell compartment. Except where noted, excitation was at the wave length corresponding to maximum emission for a given fluorophore. The emission monochromator was fitted with a synchronous drive so that reproducible emission spectra could be recorded. All the spectra reported are uncorrected but were related to appropriate standards for evaluation. Fluorescence intensities measured with the associated photomultiplier microphotometer were recorded on a Varian G-11 recorder.

Fluorescence Polarization Measurements—The Amino-Bowman spectrophotofluorometer was modified for polarization measurements by the introduction of Glanprism polarizers into the exciting and emitted beams. The transmission axes of these polarizers could be rotated to allow the parallel and perpendicular polarization components of the incident and emitted light to pass. The procedure for measuring the polarization, \( P \), consists of making four intensity measurements, one for each pair of orientations of the exciting and analyzing prisms. The value derived experimentally for \( P \) is given by

\[
P = \frac{I_{BB} - I_{EB}}{I_{BB} + I_{EB}} \left( \frac{I_{BB}}{I_{EB}} \right)
\]

where the notation for the orientation of the polarizers is: \( E \), polarizer transmission axis perpendicular to the plane formed by the excitation beam and the observation beam; \( B \), polarizer transmission axis parallel to this plane. In any subscript pair the first refers to the excitation polarizer and the second to the analyzer. This method of measurement corrects for the degree of polarization imposed by the grating in the emission monochromator.

Equations derived by Perrin (13) and Weber (14) were used to relate \( P \) to the molecular parameters of an equivalent, rigid, randomly labeled, ellipsoidal macromolecule. Perrin’s equation,

\[
\left( \frac{1}{P} \right) - \left( \frac{1}{P_0} \right) = \frac{1}{3} \left( 1 + \frac{RT \tau}{V} \right)
\]

was assumed and a plot of \((1/P) = (1/3)) versus \( T/V \) was constructed. Here \( P \) is the measured polarization, \( R \), the gas constant; \( \tau \), the fluorescence lifetime of the fluorophore; \( V \), the viscosity; \( T \), the absolute temperature; and \( V \), the molecular volume. \( P_0 \) is the polarization observed in the absence of significant molecular rotation. The plus sign is applicable when excitation is with an unpolarized incident beam. In these studies the viscosity was varied by the use of sucrose or glycerol solutions at the constant temperature of 298°K, except where noted. For monodisperse solutions of labeled molecules, the Perrin plots were straight lines and extrapolation to \( T/V = 0 \) gave values of \((1/P_0) - (1/3))\). The harmonic mean of the rotational relaxation times corresponding to the three principal axes of the equivalent ellipsoid, \( \rho_0 \), was obtained from the relation of Debye,

\[
\rho_0 = \frac{3\eta V}{RT}
\]

corrected to the viscosity of water at 293°K and designated \( \rho_0^{20} \).

RESULTS

Nature of Active Site Sulfhydryl Group—The rhodanese monomer contains two sulfhydryl groups, of which the one more reactive with a variety of reagents is essential for the catalytic activity (6). Evidence concerning the nature of this sulfhydryl group was obtained in experiments with dodecyl sulfate. A fully active enzyme sample was assayed for sulfhydryl groups by the method outlined under “Experimental Procedure” and two sulfhydryl groups were found per monomer. The assay was repeated but the sodium dodecyl sulfate (2%) used to “disrupt the structure” was added to the enzyme before the DTN. An absorbance corresponding to between 1.0 and 1.5 sulfhydryl groups per monomer was observed, depending on the interval between the additions of SDS and DTN. Fig. 1 shows the results of an experiment in which the kinetics of color development was followed. These results may be summarized as follows. (a) DTN alone reacts slowly with one sulfhydryl group and subsequent addition of SDS reveals the other, and (b) SDS added before DTN permits only one sulfhydryl group to be detected. The data indicate that interaction of the enzyme with SDS can protect one sulfhydryl group from reaction with the DTN.

Fluorescein mercuric acetate reacts with sulfhydryl groups to give covalent conjugates. When FMA was added to a sample of rhodanese (Fig. 2), the enzymic activity fell to 60% of its control value in 1 hour. At this point mercaptoethanol was added to both sample and control. The control sample slowly lost activity due to inactivation by mercaptoethanol. In the FMA-treated enzyme, however, the activity increased for 10 min due to reversal of the FMA-enzyme reaction, followed by the slower inactivation by mercaptoethanol. As a control, FMA was previously treated with mercaptoethanol before adding it to the enzyme. There was no effect on the activity. These results indicate that FMA decreases the activity of rh-
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**FIG. 1.** Kinetics of color development in assay for sulphydryl groups. The reagent added last is indicated. The enzyme concentration in each run was 0.5 mg per ml. **Inset:** Effect of adding SDS to an enzyme sample that already contained DTN.

**FIG. 2.** FMA inactivation of rhodanese. ○—○, activity of control to which no FMA was added; Δ—Δ, activity of sample which was 10⁻⁴ M in FMA; ⨿—-, activity of sample relative to control. Mercaptoethanol for reversal was 0.14 M. The experiment was run at pH 10.0 in glycine buffer, ionic strength = 0.2. Mercaptoethanol was added at 60 min.

To evaluate the nature of the region surrounding the active site sulphydryl group, the ultraviolet spectrum of FMA-inactivated rhodanese was investigated. The results are shown in Fig. 3 as a difference spectrum. Curve 1 shows the difference spectrum of FMA in methanol against FMA in H₂O. Curve 2 shows the difference spectrum of rhodanese plus FMA against rhodanese plus FMA-mercaptoethanol. The FMA difference spectrum in Curve 1 arises from the characteristic long wave length shift of π→π* transitions on going from aqueous solution to a solvent of higher polarizability. The difference spectra 1 and 2 obtained in this way are very similar.

**Hydrophobic Probing Reagents**—To test the possible involvement of identifiable “hydrophobic” regions on the enzyme, a series of experiments was performed with the fluorescent mole-

cule 2-p-toluidinynaphthalene-6-sulfonate. TNS is practically nonfluorescent in aqueous solution but its intensity increases markedly in media of low dielectric constant and when associated with proteins known to possess hydrophobic binding sites (12, 16). In Fig. 4 are reproduced the spectra obtained with TNS in aqueous solution before and after addition of an aliquot of rhodanese and a 10-min incubation. There was a large increase in fluorescence intensity and the spectrum with rhodanese was very similar to that obtained with TNS in methanol. As reported by McClure and Edelman, the intensities were found not to be related to the solvent viscosity (12).

In experiments involving the binding of TNS to rhodanese it was found that prior incubation of the enzyme with the reagent was necessary before a reproducible spectrum or reading could be taken. Following addition of TNS, there was a fluorescence increment that was faster than the time constant for measurement, followed by a slower increase in intensity. The velocity...
of this slower phase of TNS binding was sensitive to ionic strength and to the presence of SDS at concentrations (0.2%) found insufficient alone to cause increased TNS fluorescence. An experiment in which the fluorescence polarization of the fluorescein isothiocyanate conjugate of rhodanese (see below) was followed after the addition of SDS also showed a decrease in polarization with time. However, when the rhodanese used in TNS tests had been inactivated with dinitrobenzene under conditions previously shown to produce an oxidized dimer by formation of an intermonomer disulfide bridge, only the instantaneous fluorescence increment was retained; there was no longer a slow increase even in the presence of SDS.

Covalent Fluorescent Labeling of Rhodanese—Rhodanese was labeled with fluorescein isothiocyanate in phosphate buffer, pH 7.4, saturated with the competitive inhibitor, naphthalene-sulfonate, to slow reaction at the active site. By restricting labeling time to 4 min under those conditions it was possible to obtain a labeled enzyme that retained a high specific activity (>90% of that of the crystalline enzyme). Extending the reaction time to 1 hour gave a small further increase in dye output on being transferred from aqueous solution to media of low dielectric constant and to the presence of SDS at concentrations (0.2%) found insufficient alone to cause increased TNS fluorescence. An experiment in which the fluorescence polarization of the fluorescein isothiocyanate conjugate of rhodanese was followed after the addition of SDS also showed a decrease in polarization with time. However, when the rhodanese used in TNS tests had been inactivated with dinitrobenzene under conditions previously shown to produce an oxidized dimer by formation of an intermonomer disulfide bridge, only the instantaneous fluorescence increment was retained; there was no longer a slow increase even in the presence of SDS.

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Covalent fluorescent labeling of rhodanese (Rhod). Δ—ΔΔ, fluorescein isothiocyanate-labeled rhodanese inactivated by treatment with o-dinitrobenzene; •—•, fluorescein isothiocyanate-labeled rhodanese; ○—○, DNS-labeled rhodanese inactivated by air oxidation. All runs were performed in phosphate buffer, pH 7.6, with a protein concentration of 0.1 mg per ml. FITC, fluorescein isothiocyanate.

Discussion

The essential nature of one of the two sulfhydryl groups present in the rhodanese monomer was shown by previous work (6) based on inactivation of the enzyme with alkylating agents, aromatic nitro compounds, and aliphatic mercaptans. Aromatic competitive inhibitors for the substrate thiosulfate also protected the reactive sulfhydryl group from attack. On the other hand, aromatic oxidizing agents (e.g. dinitrobenzene) were especially reactive with the critical sulfhydryl group. These observations led to the inference that the reactive sulfhydryl group is in or near the active site.

The possibility of reacting the essential sulfhydryl group selectively has now permitted investigation of some aspects of the microenvironment surrounding the region of the active site. The results of experiments with SDS and DTN indicate that SDS can also protect the active site sulfhydryl group from attack and introduce the possibility of an active site region that can participate generally in hydrophobic interactions. Further, the difference spectrum of FMA-inactivated rhodanese versus FMA-mercaptoethanol plus rhodanese (to blank out nonspecific interactions with FMA) indicate that the FMA held at the active site sulfhydryl group acts as if it were in a region of low dielectric constant.

The experiments with TNS support and extend these observations. TNS was chosen for its ability to increase fluorescence output on being transferred from aqueous solution to media of low dielectric constant and for its close similarity to both the substrate thiosulfate and the competitive inhibitor naphthalenesulfonate. The results indicate that a hydrophobic binding site for TNS is present on the enzyme molecule. Moreover, active rhodanese treated with TNS shows a rapid initial rise in fluorescence followed by a slower phase of increase. In dimer inactivated by oxidation of the active site sulfhydryl groups, there are no slow phase kinetics even in the presence of SDS. These results appear to indicate that dissociation of the dimer, in which the active site sulfhydryl groups are apposed, also exposes TNS binding sites. This interpretation is also in accord with the results of experiments in which active fluorescein isothiocyanate-rhodanese was treated with TNS; the fluorescence polarization fell as the TNS slow phase kinetics progressed.

The work reported previously indicated that rhodanese of 37,000 molecular weight is a dimer which under appropriate conditions can be dissociated to monomers (5, 17). It was pre-
viously found that rhodanese of 37,000 molecular weight contains two active sites (18, 19). Results of a peptide map analysis showed that the dimer consists of identical subunits (5). Ultra-centrifugation and gel filtration studies indicated that SDS-treated enzyme exists as a monomer while rhodanese oxidized by air or by dinitrobenzene is a dimer (5, 6). In the present work the fluorescence polarization of air-oxidized DNS-rhodanese and dinitrobenzene-inactivated fluorescein isothiocyanate-rhodanese gave $\rho_\lambda$ values that support the conclusion that rhodanese so treated exists as a stable dimer. Moreover, when fluorescein isothiocyanate-rhodanese was treated with SDS under conditions known to produce monomer (6), the polarization fell, while similar treatment of dinitrobenzene-inactivated rhodanese showed no change in polarization. The evidence supports the conclusion that native rhodanese can exist as a dimer that can be dissociated by SDS treatment. This general phenomenon, involving the appearance of hydrophobic surfaces upon dissociation of an enzyme to subunits, was previously studied by Anderson and Weber with lactate dehydrogenase (20).

REFERENCES
1. Horowitz, P., and Westley, J., Fed. Proc., 27, 772 (1968).
2. Villarejo, M., and Westley, J., J. Biol. Chem., 238, PC1185, 4016 (1963).
3. Sorbo, B. H., Acta Chem. Scand., 7, 1129 (1953).
4. Westley, J., and Green, J. R., J. Biol. Chem., 234, 2325 (1959).
5. Volini, M., DeToma, F., and Westley, J., J. Biol. Chem., 242, 5220 (1967).
6. Wang, S.-F., and Volini, M., J. Biol. Chem., 243, 5465 (1968).
7. Davidson, R., and Westley, J., J. Biol. Chem., 240, 4463 (1965).
8. Mintel, R., and Westley, J., J. Biol. Chem., 241, 3381 (1966).
9. Horowitz, P., and DeToma, F., J. Biol. Chem., 245, 684 (1970).
10. Zamenhof, S., in S. P. Coldowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. 8, Academic Press, New York, 1965, p. 702.
11. Ellman, G. L., Arch. Biochem. Biophys., 82, 70 (1959).
12. McClure, W. O., and Edelman, G. M., Biochemistry, 5, 1908 (1966).
13. Perrin, F., J. Physique, 7, 390 (1926).
14. Weber, G., Biochem. J., 51, 155 (1952).
15. Stenke, R. F., and Edelhoch, H., Chem. Rev., 62, 457 (1962).
16. Winkler, M., J. Mol. Biol., 4, 118 (1962).
17. Volini, M., and Westley, J., J. Biol. Chem., 241, 5168 (1966).
18. Green, J. R., and Westley, J., J. Biol. Chem., 236, 3047 (1961).
19. Westley, J., and Nakamoto, T., J. Biol. Chem., 237, 546 (1962).
20. Anderson, S., and Weber, G., Arch. Biochem. Biophys., 116, 207 (1966).
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