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DENATURATION-INDUCED DISULFIDE FORMATION IN THE ENZYME RHODANESE

RICHARD D. BAILLIE and PAUL M. HOROWITZ

Dartmouth College, Department of Chemistry, Hanover, N. H. 03755 (U.S.A.)

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Summary

The effect of denaturants on the quantitation of free sulfhydryl groups in the enzyme rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) has been reinvestigated in some detail. The sulfhydryl assay with the colorimetric reagent 5,5'-dithio-bis(2-nitrobenzoic acid) Nbs_2 shows four sulfhydryl groups per enzyme molecule (mol. wt. 32 600) when the colorimetric reagent is added to the assay mixture before the denaturant, sodium dodecyl sulfate. On the other hand, only two sulfhydryl groups per molecule are observed when Nbs_2 is added after denaturation has been initiated. The time dependence observed in this latter procedure indicates that the loss of the two groups is rapid and permanent.

The results depend on the denaturant used: urea acts like sodium dodecyl sulfate while guanidine reveals four sulfhydryl groups independent of reagent order.

The assay also gives four sulfhydryl groups independent of reagent order with urea or sodium dodecyl sulfate under conditions which are expected to limit metal ion-catalyzed oxidation of sulfhydryl groups (e.g. oxygen exclusion or metal ion chelation).

Recent studies have shown that rhodanese has a molecular weight of 32 600, no disulfides and four sulfhydryl groups per molecule. These results together with the observations reported here are taken to indicate that a disulfide can be formed during denaturation of rhodanese and that the pathway of denaturation determines the result obtained.

Introduction

Previous measurements of the sulfhydryl content of the enzyme rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) were interpreted on the basis of nu-

Abbreviations: Nbs_2, Ellman's reagent, 5,5'-dithio-bis(2-nitrobenzoic acid).

merous reports that rhodanese had a single polypeptide chain molecular weight of 18 500 [1—3]. The cysteine residues were quantitated by the method of Ellman [4] using sodium dodecyl sulfate to "denature" the enzyme after the introduction of the colorimetric reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (Nbs_2) The results indicated that rhodanese contained two cysteine residues and no cystine. In addition, one of the sulfhydryl groups was shown to be essential for activity [5]. A later study showed that reversal of the order of addition of Nbs_2 and sodium dodecyl sulfate resulted in the "masking" of one sulfhydryl group [6]. It was postulated that the interaction of sodium dodecyl sulfate with a strongly apolar environment could protect one sulfhydryl group from reaction with subsequently added Nbs_2.

Several recent studies using sodium dodecyl sulfate-polyacrylamide gel electrophoresis have revealed that rhodanese prepared by the method most widely used at present [7] consists of a single polypeptide chain with a molecular weight greater than 32 000 [8—10]. In addition recent X-ray crystallographic studies are clearly consistent with the single polypeptide chain picture of the enzyme [11]. Rhodanese as now purified has the same catalytic parameters as previously reported [7]. These results suggest that previous explanations of the sulfhydryl masking effect of sodium dodecyl sulfate may be inadequate and have prompted us to reinvestigate this phenomenon.

Methods

Crystalline rhodanese was prepared essentially by the method of Horowitz and DeToma [7]. The purified enzyme as used had a specific activity of 0.67 I.U./μg or greater. Enzyme assays were performed as described by Wang and Volini [12]. Protein concentrations were determined by a modified biuret method [13] with crystalline bovine serum albumin as a standard.

The free sulfhydryl groups were assayed according to the method of Ellman [4] as modified by Wang and Volini [5]. The normal assay procedure for free sulfhydryl groups consisted of the addition of 0.1—0.6 mg protein in 0.5 ml of distilled water to an equal volume of 0.01 M Nbs_2 in 0.1 M Tris, pH 8.0. The mixture was incubated at room temperature for 15 min before the addition of 2.0 ml 3% sodium dodecyl sulfate in 0.25 M Tris, pH 8.5. In the reversed assay procedure the order of addition of the Nbs_2 solution and the sodium dodecyl sulfate buffer was interchanged. The absorbances of all assays were determined at 412 nm versus an appropriate blank. A molar extinction coefficient of 12 500 was used to convert the absorbance to sulfhydryl concentration. When using other denaturants the sodium dodecyl sulfate buffer was replaced by 8—10 M urea in 0.05 M Tris, pH 8.5, or 3.6—6.2 M guanidine in 0.05 M Tris, pH 8.5.

Deoxygenation was accomplished by bubbling N_2 through the appropriate solutions for 3 h or more. Antifoam was added to sodium dodecyl sulfate solutions. In some cases 1 part 30% sodium dodecyl sulfate solution was added to 9 parts of deoxygenated Tris buffer to yield the "3% sodium dodecyl deoxygenated buffer".

Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn [14]. Standards used to determine the molecular weight

were ovalbumin (43 000), chymotrypsinogen (25 700), carbonic anhydrase (29 000), yeast alcohol dehydrase (37 000) and lysozyme (14 400).

All absorbances were determined on a Cary 14 recording spectrophotometer with a 0.1 absorbance slidewire or a Bausch and Lomb Spectronic 20.

Results

Denaturation with sodium dodecyl sulfate

Rhodanese was assayed for free sulfhydryl groups using the normal and reversed assay procedures described in Methods. For purposes of computation the extinction coefficient of the Nbs_2 anion was assumed to be the same in all the denaturing solutions. However, it is often most useful to simply compute the ratio (R) of the observed absorbances of the normal to the reversed assay procedure. The normal assay in 2% sodium dodecyl sulfate solutions indicated 3.9 free sulfhydryl groups per molecule (Table I). This is in agreement with several amino acid analyses [15,16]. When the reversed assay was performed only 2.2 sulfhydryl groups per molecule were measured (Table I).

It was of interest to determine whether the apparent sulfhydryl group masking was due to a difference in rate of reaction with Nbs_2. Fig. 1 shows the number of free sulfhydryl groups per molecule in sodium dodecyl sulfate incubation solutions as a function time. In the upper curve (see Fig. 1 for conditions) the enzyme, as a control, was incubated in a Tris buffer, pH 8.5. The lower curve illustrates the loss of two sulfhydryl groups per molecule when the enzyme was incubated in a 2% sodium dodecyl sulfate solution. Aliquots of both incubation mixtures were assayed by the normal assay procedure at the times indicated. The small time-dependent decay of free sulfhydryl groups in both

TABLE I

MOL OF FREE SH GROUPS/MOL PROTEIN

R is the ratio of the number of free sulfhydryl groups per molecule in the normal assay procedure to the reversed assay procedure. This ratio is of the individual trials rather than the ratio of the final average values. Number of trials in parentheses.

Conditions	Normal	Reversed	*R*
2% sodium dodecyl sulfate	3.9 ± 0.7 * (17)	2.2 ± 0.5 (20)	1.9 ± 0.2 (16)
— EDTA	3.7 ± 0.6 (2)	1.9 ± 0.3 (2)	2.0 ± 0.1 (2)
+ EDTA **	3.7 ± 0.7 (4)	3.9 ± 0.6 (3)	1.0 ± 0.0 (3)
+ O_2	4.3 ± 0.2 (5)	2.4 ± 0.4 (5)	1.9 ± 0.2 (5)
— O_2	4.2 ± 0.2 (8)	3.3 ± 0.6 (8)	1.3 ± 0.2 (8)
6.4—6.8 M urea — EDTA	n.c.	n.c.	1.7 ± 0.3 (6)
6.4—6.8 M urea + EDTA	n.c.	n.c.	1.3 ± 0.1 (2)
3.1—5.5 M guanidine · HCl	n.c.	n.c.	1.1 ± 0.0 (5)
4.1 M guanidine · HCl, $2 \cdot 10^{-4}$ M $FeCl_3$	3.4	3.4	1.0
2% sodium dodecyl sulfate + O_2 ***	3.2	3.2	1.0

* Standard deviation = $[\Sigma(\text{deviation from the mean})^2/(\text{trials} - 1)]^{1/2}$

** EDTA concentration was at a 100-fold molar excess over protein.

*** 2% sodium dodecyl sulfate + O_2 indicates the process of denaturing the enzyme in the absence of oxygen then performing the normal and reversed assays using regularly oxygenated buffers.

n.c., not calculated.

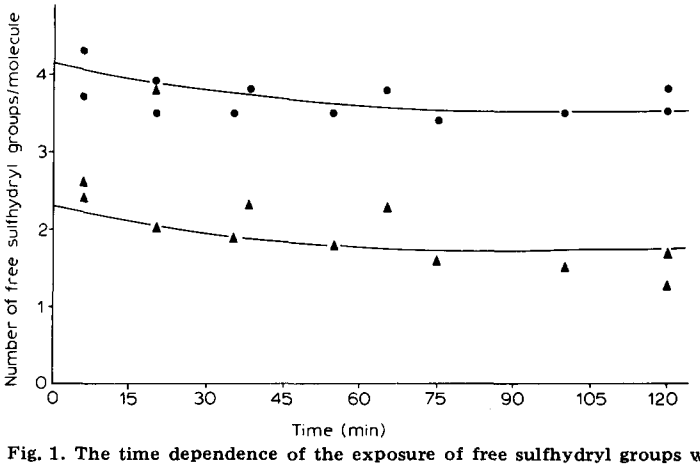

Fig. 1. The time dependence of the exposure of free sulfhydryl groups when rhodanese is incubated in a 2% sodium dodecyl sulfate solution. Rhodanese concentration in both curves is 0.47 mg protein/ml in 0.20 M Tris, pH 8.5 (upper curve) or 0.20 M Tris, 2% sodium dodecyl sulfate, pH 8.5 (lower curve). Aliquots of both incubation mixtures assayed according to the normal procedure. The upper curve is drawn to fit the experimental points. The lower curve is a tracing of the upper curve displaced so that at long times the ratio of these curves is 2.0. These curves are for comparison purposes and display the relationship expected if the ratio of forward to reverse sulfhydryl assays were exactly 2.0.

incubation mixtures may be attributed to the usual slow oxidation of the remaining free sulfhydryl groups.

Denaturation with urea or guanidine

According to the idea that the active site sulfhydryl residue is prevented from reacting with Nbs_2 by the specific binding of sodium dodecyl sulfate, other denaturants should not duplicate the anomalous results seen in the reversed assay procedure. When a concentrated solution of urea was used the ratio (R) of the observed absorbances for the normal to reversed assay was 1.7 (Table I). This was very similar to the result of 1.9 obtained for sodium dodecyl sulfate solutions. Guanidine solutions on the other hand gave only a slight difference between the two assay procedures, the ratio being 1.1.

Effect of metal ions and oxygen

The stoichiometry of the rapid loss of sulfhydryl groups suggests the possibility of disulfide bond formation. To investigate this possibility the response of the effect to metal ions and molecular oxygen was studied.

In sodium dodecyl sulfate or concentrated urea solutions the addition of EDTA eliminated the effect observed in the reversed assay (Table I). In the sodium dodecyl sulfate solution addition of EDTA caused the number of free sulfhydryl groups per molecule to increase from 1.9 to 3.9 thereby decreasing the ratio of the normal assay to the reversed assay from 2.0 to 1.0. For concentrated urea solutions this ratio decreased from 1.7 to 1.3. To ensure that these observations were a function of the denaturants used and not metal ion contamination of the sodium dodecyl sulfate and urea solutions, forward and reverse assays were performed using as denaturant 4.1 M guanidine (Methods) which was made $2 \cdot 10^{-4}$ M in $FeCl_3$. The ratio of the normal assay to reversed

assay was 1.0 (Table I). This value is in close agreement with that obtained with guanidine unsupplemented with metal ions.

To test for enzyme stability under these experimental conditions a 0.5 mg protein/ml rhodanese solution consisting of 0.1 M Tris, 1 mM EDTA, pH 8.0, was kept at 4°C and assayed for activity periodically. The activity of this sample and a control (identical solution without EDTA) remained invariant for more than 27 h.

By excluding oxygen from all the buffers before their use, the ratio for the sodium dodecyl sulfate solutions again decreased from 1.9 to 1.3. The high value of R for the urea plus EDTA and sodium dodecyl sulfate minus O_2 assays may be attributed to incomplete metal ion chelation or incomplete purging of oxygen, respectively.

In a similar experiment a sample of enzyme (15 mg protein/ml) was incubated in 3% deoxygenated sodium dodecyl sulfate for 1 h at room temperature before the normal and reversed assays were performed with regularly oxygenated buffers (last entry in Table I). The minor loss of sulfhydryl groups may be expected due to the small amount of oxygen in the original enzyme stock mixture.

Effect of pH on the reversed assay

The effect of pH on the reversed assay was determined at pH 5.0. Rhodanese as the sulfur-substituted intermediate was dissolved in 0.1 M Tris/acetate, pH 5.1, at a concentration of 1.2 mg protein/ml. To 0.5 ml of the protein solution, 2.0 ml 0.1 M Tris/acetate, 3% sodium dodecyl sulfate, pH 5.0, was added and incubated for 15 min at room temperature. The pH was readjusted to 8.5—8.6 by adding with vigorous mixing 0.10 ml 2.1 M NaOH. The addition of 0.5 ml 0.01 M Nbs_2 in 0.1 M Tris, pH 8.0, followed. The pH of the protein stock solution was readjusted to 8.0—8.5 and the normal and reversed free sulfhydryl assays were performed in the usual manner using 0.1 M Tris/acetate, 3% sodium dodecyl sulfate, pH 8.5, as the denaturing medium. The results are shown in Table II. There seemed to be no irreversible effect of the pH adjustments on the enzyme since the controls indicated the typical sulfhydryl assay results.

Effect of rhodanese concentration on the reversed assay

A major difference between the normal and reversed assay conditions is the protein concentration during the incubation period. The normal assays were incubated at a protein concentration of 0.1—0.6 mg/ml while the reversed assays had a protein concentration 2.5 times less than that of the normal assay. To test the effect of this difference, various amounts of a 38.6 mg/ml protein solution of rhodanese was assayed for free sulfhydryl groups by the reversed assay procedure. The amount of protein in the reversed assay incubation mixture (0.08—0.46 mg/ml) overlapped the protein concentration commonly obtained in the normal assay incubation mixture (0.38 mg/ml). There was a linear response of the reversed assay for free sulfhydryl groups to the total amount of protein in the assay mixture. The number of free sulfhydryl groups per molecule by the reversed assay procedure ranged from 2.5 to 2.8 in this experiment. A normal assay for free sulfhydryl groups was 4.3 free sulfhydryl groups per molecule. Therefore, R equals about 1.7 for this particular enzyme sample.

TABLE II

EFFECT OF pH ON THE REVERSED ASSAY

The results are expressed as mol of free SH groups/mol protein.

Conditions	Normal	Reversed	R *
pH 5.0	n.d.	2.3	n.d.
pH 8.5	3.7	2.0	1.9

* Same as for Table I.
n.d., not determined.

Sodium dodecyl sulfate gel electrophoresis

To obtain physical evidence for a disulfide bridge in appropriately denatured forms of rhodanese, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used. Samples of the sodium dodecyl sulfate assay solutions for free sulfhydryl groups were immediately prepared for electrophoresis by the addition of bromphenol blue and solid sucrose. Microliter amounts were added to the gels and electrophoresed in the constant current mode. There were no differences observed in the relative mobilities of the enzyme fully labelled with Nbs_2 as compared to the form containing the proposed disulfide bond. All the samples were consistent with a molecular weight of 31 000—33 000. Only a very faint higher molecular weight band was seen in some gels.

Discussion

The abnormal reactivity of sulfhydryl groups in rhodanese induced by sodium dodecyl sulfate denaturation was investigated in response to the recent reports that rhodanese is actually a polypeptide chain with a molecular weight of 32 000—35 000. This information raises new alternatives to the previous interpretation of the sulfhydryl "masking" effect of sodium dodecyl sulfate with rhodanese. The model was dependent on the loss of a single sulfhydryl residue in a low molecular weight form of the enzyme (18 500), along with the consideration that the detergent could bind tightly to the highly hydrophobic region of the enzyme's active site [6]. It was presumed that in the presence of the Nbs_2 the solvent accessible sulfhydryl group is labelled and the subsequent addition of sodium dodecyl sulfate reveals the second group in the monomer. In the absence of Nbs_2 the enzyme is denatured, resulting in a sodium dodecyl sulfate-protected sulfhydryl group and an exposed group ready for labelling with the sulfhydryl reagent.

Amino acid analyses of rhodanese indicate four half-cystine residues per molecule with a molecular weight of approx. 33 000. There has been no evidence to date for the presence of a disulfide bridge in the native structure. We have confirmed that the total number of free sulfhydryl groups per molecule as determined by chemical modification with Nbs_2 depends on the order of addition of the denaturant and sulfhydryl reagent to the protein solution. The maximum number of free sulfhydryl groups seen using sodium dodecyl sulfate as the denaturant is approximately four per molecule, and this number decreases to about two per molecule under the reversed assay conditions. Upon ex-

panding the investigation to other denaturants identical results were obtained with urea, but with guanidine solutions the assay gives four sulfhydryl groups per molecule independent of the order of addition of the reagents.

It is evident from the stoichiometry of the loss of the free sulfhydryl groups that the formation of a disulfide bond may result from denaturation of rhodanese by specific agents. The model of special sodium dodecyl sulfate protection of two free sulfhydryl groups per molecule from reaction with Nbs_2 can be eliminated on the basis of the results obtained with concentrated urea. The formation of this proposed disulfide bond may be inhibited by blocking the usual metal ion-catalyzed oxidation process by either the removal of molecular oxygen from the buffer system or the chelation of free metal ions in solution. In addition the results in Table II illustrate that even well below the pK_a of a solvent accessible sulfhydryl group (pK_a = 8.3, cf. ref. 17), a disulfide bond may still be formed during denaturation.

Sodium dodecyl sulfate gel electrophoresis was used to try to differentiate the two species produced in the assays on the basis of the different shapes and amount of sodium dodecyl sulfate bound per molecule. The fact that no difference in the relative mobilities of the two species was observed does not discount the presence of the proposed disulfide bridge. The same result has been seen for lysozyme when comparing the fully reduced protein with the unreduced protein containing four disulfide bridges [18]. However, ovalbumin, containing one disulfide bridge, has a marked difference in the relative mobilities of reduced and unreduced forms on sodium dodecyl sulfate gels. These results have been confirmed in this laboratory.

As discussed in an earlier section, when the enzyme is fully denatured in sodium dodecyl sulfate solutions in the absence of molecular oxygen, its full complement of free sulfhydryl groups is measured, even if oxygen is subsequently admitted. Since the effect cannot be ascribed either to the initial conformation or the final denatured state of the enzyme, it appears that the pathway of denaturation is an important factor in forming a disulfide bridge in the enzyme. The comparison of the effect using guanidine supplemented with Fe^{3+} further supports the idea that the results obtained depend on the nature of the denaturation process and not primarily on possible metal ion contamination in the denaturant solutions used. In addition, it is tempting to speculate that the two sulfhydryl groups involved may be spatially oriented quite close to each other in the enzyme's tertiary structure.

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