Oxidative Inactivation of the Enzyme Rhodanese by Reduced Nicotinamide Adenine Dinucleotide*

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The enzyme rhodanese (thiosulfate sulfurtransferase; EC 2.8.1.1) is inactivated with a half-time of approximately 3 min when incubated with 50 mM NADH. NAD+, however, has virtually no effect on the activity. Inactivation can be prevented by the inclusion of the substrate thiosulfate. The concentration of thiosulfate giving half-protection is 0.038 mM. In addition, NADH, but not NAD+, is a competitive inhibitor with respect to thiosulfate in the catalyzed reaction ($K_i = 8.3$ mM). Fluorescence studies are consistent with a time-dependent oxidation of NADH in the presence of rhodanese. The sulfur-free form of rhodanese is more rapidly inactivated than the sulfur-containing form. Spectrophotometric titrations show that inactivation is accompanied by the loss of two free SH groups per enzyme molecule. Inactivation is prevented by the exclusion of air and the inclusion of EDTA (1 mM), and the enzyme activity can be largely protected by incubation with superoxide dismutase or catalase. Rhodanese, inactivated with NADH, can be reactivated by incubation with the substrate thiosulfate (75 mM) for 48 h or more rapidly, but only partially, by incubating with 180 mM dithiothreitol. It is concluded that, in the presence of rhodanese, NADH can be oxidized by molecular oxygen and produce intermediates of oxygen reduction, such as superoxide and/or hydrogen peroxide, that can inactivate the enzyme with consequent formation of an intraprotein disulfide. In addition, NADH, but not NAD+, can reversibly bind to the active site region in competition with thiosulfate. These data are of interest in view of x-ray studies that show structural similarities between rhodanese and nucleotide binding proteins.

The enzyme rhodanese (thiosulfate cyanide sulfurtransferase; EC 2.8.1.1) catalyzes the transfer of the outer sulfur of thiosulfate to a variety of nucleophilic acceptors such as cyanide and can provide the labile sulfur that is part of the characteristic prosthetic group of the iron-sulfur proteins (1, 2). In the course of these reactions, the enzyme cycles through two stable catalytic intermediates: the sulfur-free form ($E^*$) and the sulfur substituted form ($ES$) (3).

X-ray crystal studies of the $ES$ complex of rhodanese have shown (4, 5) that the 299-residue single polypeptide chain is folded into two equal size domains that are connected by a single strand of polypeptide chain. The active site is in a region formed by the juxtaposition of the two domains, and the sulfur atom transferred during catalysis is bound in a persulfide linkage to cysteine 247 at the active site. The double-domain structure is stabilized mainly by hydrophobic interactions which may also provide an appropriate environment for catalysis. In view of the effectiveness of aromatic substrates, there has been some interest in binding hydrophobic anions in this region of the protein (6). The x-ray results further suggested that rhodanese might contain a "dinucleotide fold," and the order of the parallel B-strands were reminiscent of that found in the nucleotide binding domains of dehydrogenases and in flavodoxin (7–9). Binding of nucleotides to crystalline rhodanese was attempted by converting the crystalline $ES$ form to $E$ and soaking the resulting crystal for 5 weeks in solutions containing 50 mM nucleotide. No nucleotide binding to the crystals was observed, but it was reported that there might have been oxidation of $S(y)$ of the active site sulfhydryl group on cysteine 247 to the sulfinic acid (7). There are several reasons, though, why nucleotide binding might occur with the soluble enzyme: (a) the binding might be weak and not observable under the conditions used to maintain the crystal; (b) there is some evidence that the solution structure of rhodanese differs somewhat from that in the crystal, and some conformational changes are inhibited by the crystal environment (10); and (c) the $E$ form of the enzyme is known to be labile (11). It is interesting in this regard that early suggestions were made that binding to immobilized Cibacron blue F3GA might be diagnostic for proteins with a dinucleotide fold (12), and rhodanese was found to bind to such supports. In fact, an effective purification of the protein was designed on this basis (13). Accumulating evidence, though, showed that a dinucleotide fold is a sufficient but not necessary condition for binding to these dyes (14, 15).

We report here the effects of nicotinamide adenine dinucleotides on rhodanese in solution. It is concluded that there is weak preferential binding of NADH as compared with NAD+. Further, a low level of NADH oxidation occurs, apparently giving rise to partial reduction products of molecular oxygen (hydrogen peroxide and superoxide) that can inactivate the enzyme.

**MATERIALS AND METHODS**

All reagents used were analytical grade. Reduced and oxidized forms of $\beta$-nicotinamide adenine dinucleotide (NADH and NAD+) were grade III from yeast and obtained from Sigma. Superoxide dismutase was from bovine erythrocytes (Sigma S 8254), and catalase was obtained as a 2x crystalline suspension (Sigma C 100). Unless otherwise specified, the buffer used throughout was 100 mM sodium dihydrogen phosphate, pH 7.0.

Rhodanese was prepared as previously described (13) and stored in the sulfur-containing $ES$ form at $-70^\circC$ as a crystalline suspension.

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1 The abbreviations used are: $E$, the sulfur-free form of rhodanese; $ES$, the sulfur-substituted form of rhodanese.
in 1.8 M ammonium sulfate. Protein concentrations were determined using a value of $A_{280}^{\text{nm},\text{m}} = 1.75 \text{ (16).}$ Rhodanese activity was assayed by a discontinuous method that quantitates the product, thiocyanate, based on the absorption of the ferric thiocyanate complex (17). The sulfur-free form ($E$) was prepared by adding a small molar excess of cyanide to the ES form as previously described (18). The ES form was used unless otherwise specified. Further details are presented under "Results and Discussion."

NADH and NAD$^+$ were tested for their effects on the rhodanese-catalyzed reaction between cyanide and thiosulfate. The assay system was at pH 8.1 and contained 500 mM glycine, 50 mM KCN, 40 mM potassium dihydrogen phosphate, and the thiosulfate concentration was varied from 2-50 mM. To test inhibition by NADH and NAD$^+$, these compounds were present at 5 mM. The rhodanese concentration in the assay was 1 $\mu$g/ml. At each thiosulfate concentration, the assay was performed at room temperature and stopped at intervals of 10, 15, 20, 30, 45, and 60 s. Initial velocities were determined from the linear portions of plots of optical density at 460 nm versus time.

Fluorescence measurements were performed in an SLM 4800 fluorimeter using an excitation wavelength of 340 nm and an emission wavelength of 438 nm.

The sulfhydryl content of rhodanese was determined by titration with 5,5'-dithiobis-(2-nitrobenzoic acid), essentially as described by Ellman (19). The protein concentration was 0.25 mg/ml, and each 2-ml assay contained 0.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and 2% sodium dodecyl sulfate. Samples were dialyzed before assay.

All other experimental details are presented in the text and figures.

**RESULTS AND DISCUSSION**

Fig. 1 shows the effect of adding NADH or NAD$^+$ on the activity of the ES form of rhodanese. NAD$^+$ was without effect, while NADH rapidly inactivated the enzyme, in this case, with a half-time on the order of 5 min. Although there was some variability in the detailed kinetics of the activity loss, presumably due to variable amounts of adventitious components in the buffers (see below), there was never any change in the general behavior described here.

Fig. 2 shows first order plots for the inactivation of rhodanese by NADH and the protection that was afforded by the substrate thiosulfate. In the presence of 50 mM thiosulfate, there was practically no inactivation of the enzyme even after 60 min of incubation with 100 mM NADH. The inset to Fig. 2 shows a double reciprocal plot relating the half-times for inactivation to the concentrations of thiosulfate. Analysis of this curve gives a value of 0.038 mM for the concentration of thiosulfate that can half-protect the enzyme against the inactivation by NADH. The $K_d$ for thiosulfate has been shown to be equivalent to its dissociation constant (3) and has been measured under similar conditions to those used here and found to have a value of 7 mM (see below and Ref. 20). No tighter binding of thiosulfate has been reported, and, therefore, it was unlikely that protection by thiosulfate against NADH inactivation was a reflection of thiosulfate binding. It was possible that the phenomenologically derived protection constant measured here was reflecting the ability of thiosulfate to act as a reducing agent and to protect against a substance produced in the presence of NADH.

**FIG. 2.** First order plot showing thiosulfate protection against inactivation of rhodanese by NADH. Rhodanese inactivation was performed as in Fig. 1 except that 100 mM NADH was used for inactivation (lowest curve, $\bullet$). The effects of the following concentrations of thiosulfate on the inactivation by NADH are shown: 0.02 mM (○), 0.059 mM (●), 0.078 mM (□), 0.625 mM (▲), and 5.0 mM (×). Inset: plot of $1/\text{inactivation half-time}$ versus $1/\text{[thiosulfate]}$. Values of the inactivation half-time are derived from first order plots of the type shown in the main figure. All other details are presented in the text.

Fig. 3 shows the time course of NADH fluorescence of 1 $\mu$M NADH when it was incubated with the E or ES forms of rhodanese. The major result was that the change in fluorescence was that expected for oxidation of a small amount of NADH, and there was no large change in the wavelength maximum or bandwidth of the NADH fluorescence spectrum as the fluorescence decrease proceeded (data not shown). It is interesting that the effect on the NADH fluorescence depended on the presence of the protein. Further, the amount of apparent NADH oxidation was limited and, therefore, the small change in NADH concentration was not observable in absorption or fluorescence if the level of NADH was raised to 50 mM as used in the inactivation experiments.

A number of inactivating reagents have differential effects on the E and ES forms of rhodanese, with ES being the more stable form (11). The results of an experiment to test this with NADH is shown in Fig. 4. The reaction was slowed by the use of lower concentrations of NADH (1 mM), and the upper curve shows that the ES activity was relatively constant for 90 min under the conditions used here. The E form, on the other hand (lower curve), showed considerable loss of activity over 90 min after an induction period of approximately 10 min. This may reflect the accumulation of an...
inactivating substance following the mixing of the components. There was no observable effect on the activity of either form of the enzyme by addition of NAD$. After 120 min, the solution of inactive enzyme was made 180 mM in dithiothreitol, and the activity rose from 15–33% over the next 45 min. This experiment is somewhat flawed by the known inactivating effect of dithiothreitol on rhodanese (21, 22). On the other hand, addition of thiosulfate to 75 mM resulted in the regain of 85% of the lost activity after incubation at 4°C for 48 h.

It is known that the sulphydryl groups of rhodanese are susceptible to reaction with consequent loss of activity, and this susceptibility is far greater for the E form. Sulphydryl titers were determined as described under "Materials and Methods" for rhodanese that had been totally inactivated with 10 mM NADH and compared with the native protein. Samples were then dialyzed for 48 h in the cold. The sample inactivated with NADH revealed 1.77 sulphydryl groups per molecule of rhodanese, while the native enzyme showed 3.6 sulphydryl groups per molecule, which is consistent with the 4 sulphydryl groups known to be present on the active enzyme (4). These results raised the possibility that rhodanese inactivation involves oxidation to form a disulfide.

It was unexpected that the reducing agent NADH would be involved in the oxidative inactivation of rhodanese, and this raised the possibility that the actual oxidizing agent might be one of the reactive intermediates that can be formed on the partial reduction of oxygen. Fig. 5 shows an experiment that supports this possibility in that it shows that superoxide dismutase (middle curve) or catalase (upper curve) can protect rhodanese against inactivation occurring in the presence of NADH (lowest curve).

Fig. 5 further shows that elimination of oxygen and chelation of adventitious metal ions had a protective effect on the activity of rhodanese. These results are consistent with the idea that NADH is involved in the formation of a partially reduced oxygen species that can inactivate rhodanese by mechanisms leading to sulphydryl oxidation.

The facts that: (a) low concentrations of thiosulfate protected rhodanese from inactivation by NADH and (b) the half-times for inactivation are long compared with the time necessary to measure the rhodanese activity (<1 min), permitted determination of the effects of NADH and NAD$^+$ on the catalyzed reaction. The results of such an experiment are shown in Fig. 7. NAD$^+$ had little, if any, effect on the activity.
The apparent $K_m$ under these assay conditions was 2.3 mM which is consistent with values previously reported (17). The upper curve shows that NADH is apparently a competitive inhibitor of the catalyzed reaction, and analysis gives a value of $K_i = 8.3$ mM. These results are consistent with previous reports that a number of aromatic anions can act as inhibitors of the rhodanese reaction in competition with thiocyanate (17, 23, 24). While it is not entirely clear why there is such a difference between NADH and NAD$,^+$, it may be related to the altered charge distribution on the NAD$^+$ since it has been shown that only the anion binds well to the protein, and charged zwitterionic molecules that are otherwise appropriate will not act as substrates or inhibitors (24). Thus, binding of NAD$^+$ may be precluded because of the unfavorable energetics of bringing a positively charged group into the hydrophobic part of the active site.

We conclude that rhodanese can be rapidly inactivated by products that form in the presence of oxygen, NADH, and rhodanese itself that are probably hydrogen peroxide and/or superoxide, and may less directly involve hydroxyl radical formed from these by processes that have been discussed (25). The exact mechanism of this effect is not clear at present, although only very small quantities of the agents are produced, and it is the extreme sensitivity of rhodanese that makes them detectable. In fact, this sensitivity may make the rhodanese system a potentially useful assay method for these reduced intermediates of oxygen. These suggestions are in keeping with other studies showing the extreme sensitivity of rhodanese to intermediates in the reduction of molecular oxygen formed, for example, in the cyanide-promoted inactivation of rhodanese by ketoaldehydes (26), and in the inactivation of rhodanese by dithiothreitol (21) where the autooxidation of the thiol is involved in the formation of reactive reduced oxygen species (25). The fact that thiosulfate at very low concentrations prevents inactivation, although it binds relatively weakly at only a single site, is in keeping with the suggestion that thiosulfate protects by virtue of its ability to act as a reducing agent rather than as a substrate.

REFERENCES