Biochimica et Biophysica Acta, 485 (1977) 95—100

BBA 68247

STUDIES OF THE *N*-BROMOSUCCINIMIDE INACTIVATION OF THE ENZYME RHODANESE

KATHLEEN GUIDO and PAUL HOROWITZ

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284 (U.S.A.)

(Received April 12th, 1977)

Summary

The enzyme rhodanese (Thiosulfate : cyanide sulphurtransferase, EC 2.8.1.1) is rapidly inactivated by treatment with *N*-bromosuccinimide. Spectrophotometric titration and sodium dodecyl sulfate polyacrylamide gel electrophoresis show that neither tryptophan oxidation nor polypeptide chain cleavage can account for the inactivation. Sulfhydryl group assays using the colormetric reagent 5,5′-dithiobis(2-nitrobenzoic acid) after destruction of excess *N*-bromosuccinmide, indicate that approximately 2 sulfhydryl groups per enzyme molecule are lost. Further, rhodanese inactivated by *N*-bromosuccinimide can be reactivated (~95%) by incubation with the substrate thiosulfate. It is postulated that *N*-bromosuccinimide inactivates rhodanese by inducing the formation of a disulfide bond involving the active site sulfhydryl group of the enzyme and a second sulfhydryl group which can be brought close to the active site in the flexible native structure.

Introduction

The enzyme rhodanese (thiosulfate : cyanide sulfurtransferase, EC 2.8.1.1) is rapidly inactivated on the addition of stoichiometric quantities of *N*-bromosuccinimide [1,2]. The reported correlation between the loss of enzyme activity and the loss of tryptophan was the primary basis for the implication of this residue as an active site component [1]. The secondary evidence for this conclusion has recently been the subject of reinvestigation and reinterpretation [3—5]. Preliminary experiments using *N*-bromosuccinimide in our laboratory did not show the expected loss of tryptophan [2]. The active site of rhodanese is known to contain at least one free essential sulfhydryl group [6] and its

Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate.

oxidation by *N*-bromosuccinimide would be in keeping with the relatively broad reactivity of this reagent [7].

We report here the details of studies of the effect of NBS on rhodanese to further elucidate the mechanism for the observed inactivation.

Materials and Methods

Crystalline bovine liver rhodanese (mol. wt. 32 600) was prepared by the method of Horowitz and DeToma [8] with minor modifications and stored at −70° C as an ammonium sulfate suspension. *N*-bromosuccinimide was obtained from Nutritional Biochemicals Corp., recrystallized from water; then dried and stored in a vacuum dessicator. The *N*-bromosuccinimide was recrystallized the day before use. All other materials were the best available commercial grades and were used as obtained.

Enzyme activity was measured using the assay procedure of Wang and Volini [6]. Protein concentrations were measured with a modified biuret procedure [9]. Absorption measurements were made either on a Cary 14 spectrophotometer or a Beckman Model 25 recording spectrophotometer. Electrophoresis was done using an SDS-disc system modified from Maizel [10]. Sulfhydryl groups were measured by a modified Ellman's procedure [6,11]. An extinction coefficient of 12 500 was used for the reduced 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 2% sodium dodecyl sulfate (SDS).

Measurement of absorbance at 280 nm upon N-bromosuccinimide oxidation

Enzyme solutions ranging in specific activity from 0.53 to 0.84 I.U./μg were desalted on a Bio-Rad P-2 column equilibrated with 0.1 M glycine and diluted with the same buffer to approximately $3 \cdot 10^{-6}$ or $2 \cdot 10^{-5}$ M. The spectra were scanned from 250 to 360 nm using 0.1 M glycine as the blank. Between recordings, the sample was maintained at 0° C.

Titrations with a freshly prepared aqueous solution of *N*-bromosuccinimide were done in two ways: (a) 1-μl aliquots of *N*-bromosuccinimide were sequentially added to a single enzyme sample with vigorous mixing. Enzyme activity and an absorbance scan using an appropriate blank were measured immediately after each addition; (b) multiple identical enzyme samples were prepared and appropriate microliter aliquots of an *N*-bromosuccinimide solution were added to achieve a range of *N*-bromosuccinimide to rhodanese ratios. Enzyme assays were performed before and after an absorbance scan using an appropriate blank.

Tryptophan destruction was calculated from the measured decrease in absorbance at 280 nm by the method of Spande and Witkop [12,13].

Determination of cysteine content after N-bromosuccinimide oxidation

Samples of rhodanese ranging in specific activity from 0.65 to 0.71 I.U./μg were desalted as above and diluted to a concentration of 0.25 mg/ml. 1-μl aliquots of an *N*-bromosuccinimide solution were added to 2.5 ml of this solution and the activity was monitored until it had dropped to about 20% of its original value. At that point tryptophan in 0.1 M glycine was added to destroy any remaining *N*-bromosuccinimide and the pH was raised to 8 by the addition

TABLE I

Sample No.	Initial activity *	Treatment No. 1 **	Activity after * treatment No. 1	Treatment No. 2 **	Activity 48 h * after treatment No. 2
1	650	+NBS ***	421	$+S_2O_3$	564
2	650	+NBS	421	—	392
3	650	—	650	$+S_2O_3$	602
4	650	—	650	—	592

* Expressed in international units/mg protein.
** For details see Materials and Methods.
*** *N*-bromosuccinimide.

of 1 M Tris, pH 8.2. DTNB was added at a 50-fold molar excess over enzyme. After a few minutes, sufficient SDS dissolved in 0.1 M Tris, pH 8.0 was added to make the solution 2% in SDS, and thoroughly mixed. After 15 min, the absorbance at 412 nm was measured in this solution (final volume of 3.3 ml) against appropriate blanks and controls. The number of sulfhydryl groups/32 600 daltons was determined and compared for the control and sample.

In other trials the above procedure was repeated using 0.1 M Tris, pH 8.0, in place of glycine.

Reactivation of N-bromosuccinimide treated rhodanese

Samples of rhodanese (650 I.U./ml) were partially inactivated by the addition of 0.72 mol of *N*-bromosuccinimide per mol of rhodanese. After 10 minutes, to ensure that the activities reached were stable, sodium thiosulfate was added to samples and control at a 30-fold molar excess over rhodanese and the samples were incubated at 5°C. Rhodanese activities were measured as a function of time and the results obtained after 48 h are shown in Table I.

Results and Discussion

N-bromosuccinimide is known to have many effects on polypeptides, one of which is the oxidation of the indole ring of tryptophan to an oxindole [12,13] with a concommitant change in the absorption spectrum. In the case of rhodanese, it is difficult to follow the changes in absorption if a single enzyme sample is titrated because addition of *N*-bromosuccinimide, even with rapid mixing to prevent high local *N*-bromosuccinimide concentrations, produces turbidity in the solution, as noted by Davidson and Westley [1]. In the present study, an attempt was made to correct for this effect by scanning each sample from 250 to 360 nm and correcting the baseline for turbidity by the method of Donovan [23].

The activity in this titration fell as expected [1] but even when all enzyme activity had disappeared there was little or no decrease in the absorbance at 280 nm. The presence of turbidity, though, made it difficult to precisely quantitate the absorbance changes.

When the titration was performed by appropriate additions of *N*-bromosuccinimide to independent samples of rhodanese the turbidity was largely eliminated and the data in Fig. 1 was generated. Extrapolation of the rhodanese

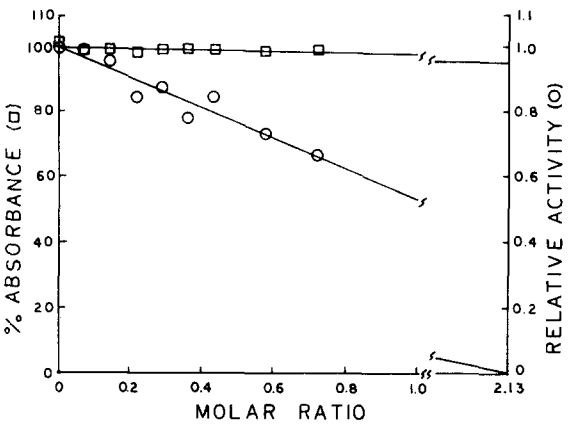

Fig. 1. Comparison of the change in rhodanese activity with the change in absorbance at 280 nm upon addition of *N*-bromosuccinimide. ○: change in activity of rhodanese samples, over the range of *N*-bromosuccinimide concentrations which produce a linear effect on the activity. The best least-squares fit of these points approaches zero activity at a molar ratio of 2.13 mol *N*-bromosuccinimide to mol rhodanese (mol. wt. = 32 600); correlation coefficient = 0.956. Rhodanese concentration: $2 \cdot 10^{-5}$ M. □: change in absorbance of samples in curve (○). The best least-squares fit of these points approaches 95.4% at a molar ratio of 2.13 mol *N*-bromosuccinimide to mol rhodanese. See text for details of sample preparation.

activity curve indicates that a maximum loss of 0.6 tryptophan residues per molecule of rhodanese would be associated with total inactivation. When this value is compared with the 2—3 tryptophan residues per molecule previously reported it is not possible to explain complete inactivation of the enzyme on the basis of tryptophan destruction.

Since *N*-bromosuccinimide can cleave peptide bonds, an attempt was made to determine whether the integrity of the polypeptide backbone of the enzyme was maintained after *N*-bromosuccinimide oxidation. SDS-disc electrophoresis in 15% polyacrylamide gels was run both on a sample of enzyme treated with *N*-bromosuccinimide to 80% inactivation and an identical sample which was left untreated. Even high degrees of protein loading (>50 μg) failed to show any cleavage bands which would be commensurate with the inactivation observed.

Since one of the reactivities of *N*-bromosuccinimide is the oxidation of cysteine [7], an experiment was undertaken to investigate whether cysteine oxidation was responsible for the activity loss. At least one of these residues has been demonstrated to be essential for rhodanese activity [6]. This was accomplished by adding sufficient *N*-bromosuccinimide to destroy approximately 80% of the sample's activity and then assaying for the content of free sulfhydryl groups.

In all trials, significantly fewer sulfhydryl groups were counted in the *N*-bromosuccinimide-treated samples than in the controls. The total number of sulfhydryl groups per enzyme molecule in the control samples ranged from 3 to 4 in five independent assays when glycine was used as the buffer. This is consistent with previous observations of low measured sulfhydryl titer in glycine solutions [15]. However, comparison of the sulfhydryl titer before and after *N*-bromosuccinimide in the present experiments shows that rhodanese inactiva-

tions is accompanied by a loss of between 2.0 and 2.4 sulfhydryl groups/ 32 600 daltons.

Previous work has shown that rhodanese can form an intraprotein disulfide involving the active site -SH group either on long storage in dilute solution in contact with air [16] or more rapidly (20 min) in the presence of stoichiometric quantities of phenylglyoxal [17]. These samples can be reactivated on long incubation with S_2O_3. Virtually instantaneous intraprotein disulfide formation has been reported to occur in rhodanese samples during denaturation with sodium dodecyl sulfate and urea [18].

Partially inactivated rhodanese was incubated with the substrate sodium thiosulfate to distinguish between the more common *N*-bromosuccinimide reactivities which result in irreversibly altered protein and an *N*-bromosuccinimide-induced disulfide formation. The results of this experiment are shown in Table I. The enzyme was inactivated to a degree (65% of the control) that would ensure that the sample would still be on the linear portion of the activity curve in Fig. 1. Inactivated samples and controls were incubated for 48 h with and without thiosulfate. In the absence of thiosulfate the *N*-bromosuccinimide treated sample (sample 2) had the same activity relative to its control (sample 4) as it had immediately after inactivation. The activity of the sample treated with thiosulfate (sample 1) had increased to 94% of its control (sample 3). That this represents a true reactivation can be seen by comparing the specific activity of sample 1 immediately after inactivation with that after 48 h and by comparing the activities of samples 1 and 2 at 48 h.

These results, taken together, are consistent with the postulate that *N*-bromosuccinimide inactivates rhodanese by inducing disulfide formation. It appears then that the active site -SH group and a second -SH group are in proximity in the native enzyme but are constrained so as to prevent facile disulfide formation. The primary structure of rhodanese [19] shows that the active site SH group (residue 247) is separated from a second cysteine (residue 254) by six residues. The recently available X-ray picture of rhodanese [20] shows that the sulfur atoms of these cysteine residues are 7 Å apart and unfavorably oriented for disulfide formation. Solution studies of rhodanese, however, consistently show that this protein has a high degree of flexibility [21,22] which is not displayed in the crystal [20]. It is tempting to speculate that the active site sulfhydryl group is structurally prevented from easily reacting but the enzyme is conformationally mobile enough to allow disulfide formation under the appropriate conditions. It is interesting that these same residues have been implicated in the observed inactivation of rhodanese induced by phenyl glyoxal (Westley, J., personal communication).

Acknowledgement

Acknowledgement is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for support of this research.

References

1 Davidson, B. and Westley, J. (1965) J. Biol. Chem. 240, 4463—4469
2 Guido, K. and Horowitz, P.M. (1976) Biophys. J. 16, 2032

3 Baillie, R.D. and Horowitz, P.M. (1976) Biochim. Biophys. Acta 429, 402—408
4 Guido, K. and Horowitz, P.M. (1975) Biochim. Biophys. Res. Commun. 67, 670—676
5 Finazzi Agro, A., Federici, G., Giovagnoli, C., Cannella, C. and Cavallini, D. (1972) Eur. J. Biochem. 28, 89—93
6 Wang, S.F. and Volini, M. (1968) J. Biol. Chem. 243, 5465—5470
7 Fontana, A. (1972) Methods Enzymol. 25, 412—423
8 Horowitz, P.M. and DeToma, F. (1970) J. Biol. Chem. 245, 984—985
9 Zamenhof, S. (1957) Methods Enzymol. 3, 696—704
10 Maizel, Jr., J.V. (1971) Methods Virol. 5, 179—246
11 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70—77
12 Spande, T.F. and Witkop, B. (1967) Methods Enzymol. 11, 506—522
13 Spande, T.F. and Witkop, B. (1967) Methods Enzymol. 11, 498—506
14 Baillie, R.D. and Horowitz, P.M. (1976) Biochim. Biophys. Acta 427, 594—599
15 Blumenthal, K. and Heinrikson, R. (1972) Biochim. Biophys. Acta 278, 530—545
16 Volini, M., DeToma, F. and Westley, J. (1967) J. Biol. Chem. 242, 5220—5225
17 Weng, L., Heinrikson, R.L. and Westley, J. (1976) Fed. Proc. 35, 1630
18 Baillie, R.D. and Horowitz, P.M. (1976) Biochim. Biophys. Acta 429, 383—390
19 Heinrikson, R.L., Russell, J., Weng, L. and Keim, P.S. (1976) Fed. Proc. 35, 1976
20 Ploegman, J.H. (1977) Ph.D. Thesis, Rijksuniversiteit Groningen
21 Volini, M. and Wang, S.F. (1973) J. Biol. Chem. 248, 7386—7391
22 Jarabak, R. and Westley, J. (1974) Biochemistry 13, 3237—3239
23 Donovan, J.W. (1969) in Physical Principles and Techniques of Protein Chemistry, Part A (Leach, S., ed.), pp. 164—165, Academic Press, New York