Inactivation of thioredoxin by sulfite ions

Martina Würfel, Ingo Häberlein and Hartmut Follmann

Fachbereich Biologie-Chemie der Universität, Heinrich-Plettstrasse 40, D-3500 Kassel, FRG

Received 5 June 1990

Oxidized thioredoxin undergoes sulfitolysis of its single disulfide bond at low concentrations of sulfite ions and protein and in the absence of denaturing agents. The reaction, which has an optimum at pH 8, was studied using [^{35}S]sulfite and *E. coli* thioredoxin as model. The product, thioredoxin-*S*-sulfonate, has a half-life of several hours in solution. It is unable to activate chloroplast NADP malate dehydrogenase. Thioredoxin sulfitolysis may therefore be a physiologically important factor in mediating the phytotoxic effects of sulfur dioxide in plants.

Enzyme activation; NADP-malate dehydrogenase; Sulfitolysis; Thioredoxin, *E. coli*; Thioredoxin-*S*-sulfonate

1. INTRODUCTION

Thioredoxins, a ubiquitous group of proteins which activate or deactivate, and thereby regulate, a wide variety of enzymes, hormones, or other proteins [1–3] possess an active site in which two cysteine residues (Cys-32 and Cys-35, in *E. coli* thioredoxin numbering) fluctuate between an oxidized disulfide and a reduced dithiol form. These two cysteines are exposed on one edge of the small (12000 Da), compact thioredoxin molecule [4]. While this molecular conformation favours the physiological reactivity and interaction of thioredoxins with their various target proteins, it will make the molecules more vulnerable to attack by sulfur- or sulfide-directed reagents and chemicals and may facilitate the inhibition of thioredoxin-regulated biochemical systems. This must be particularly serious in plant cells which contain multiple thioredoxins [5–7] and in which several enzymes of the light-activated chloroplast metabolism (e.g. fructose-bisphosphatase and NADP malate dehydrogenase) critically depend on the presence and the redox status of chloroplast thioredoxins [8–10].

We here describe such a case. Sulfur dioxide and sulfite ions are abundant, environmentally problematical, and phytotoxic compounds. Using *E. coli* thioredoxin as a model, we have observed that the oxidized protein readily reacts with sulfite ions and undergoes sulfitolysis [11,12] under near-physiological conditions. Whereas the scission of disulfide bridges in denatured proteins by 0.1 M sulfite at high pH and in the presence of oxidants is a well-known analytical method [13,14], sulfitolysis has rarely been studied in native systems, and has not been applied to thioredoxins at all. Some properties of the modified product, and biochemical consequences of the sulfite modification of thioredoxins are discussed.

Correspondence address: M. Würfel, Fachbereich Biologie-Chemie der Universität, Heinrich-Plettstrasse 40, D-3500 Kassel, FRG

2. MATERIALS AND METHODS

All chemicals and reagents were of the highest purity available. Sodium hydrogen [^{35}S]sulfite (spec. act. 10–30 mCi/mmol) and iodo[2-^{3}H]acetic acid (spec. act. 175 mCi/mmol) were obtained from Amersham-Buchler, Braunschweig. Sulfite solutions were handled and stored under nitrogen. Mono- and dicarboxymethylthioredoxins were synthesized as described [15].

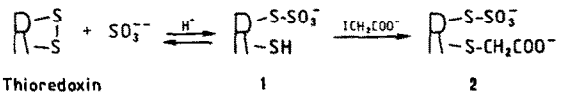
Thioredoxin isolated from *E. coli* was a product of IMCO Corp., Stockholm. NADP malate dehydrogenase (EC 1.1.1.82) was prepared from spinach leaves by the published procedure [16]. Enzyme activity was measured at 30°C and pH 7.9 as described [17]; the enzyme was activatable by μg amounts of *E. coli* thioredoxin within minutes.

In a typical sulfitolysis reaction, 40 μg thioredoxin (3.3 nmol) were dissolved in 0.33 ml 0.1 M Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, with or without 6 M guanidinium chloride, and 0.02 ml of a 0.1 M Na_2SO_3 solution (2 μmol) were added. After incubation at 25°C for the desired period of hours, 0.01 ml of 0.3 M iodoacetic acid, dissolved in Na_2CO_3 solution, were added and carboxymethylation was carried out by 2 more hours of incubation. For isolation of the unsubstituted sulfitolysis product, iodoacetate treatment was omitted but the reaction mixture cooled to 0°C and worked up immediately.

Desalting was performed on the Sephadex G-25 HR 10/10 column of a Pharmacia FPLC system, eluted with 50 mM Tris-HCl buffer (pH 8). DEAE cellulose chromatography of thioredoxins was done on DE-52 (Whatman; 1 × 2 cm) by elution with a 0–200 mM NaCl gradient in 10 mM Tris-HCl buffer (pH 8.5).

Electrophoresis on native polyacrylamide (10%) gels was done in 0.038 M glycine-Tris buffer (pH 8.9) using a Mini-Protean II system from Bio-Rad. *E. coli* thioredoxin and its carboxymethylated derivatives [15] were used as markers. Proteins were stained with Coomassie brilliant blue or by the silver method [18]. For autoradiography, electrophoresis gels were impregnated with Rotifluoroszint D [19], dried, and placed on Kodak-X-Omat AR film at −80°C.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50



Scheme 1

3. RESULTS AND DISCUSSION

The reaction between oxidized *E. coli* thioredoxin and sulfite ions was studied at low concentrations of both the protein (100–200 μg/ml) and sodium sulfite (2–6 mM) in the absence and presence of guanidinium chloride as denaturant. Formation of the thiosulfonate product in the sulfitolysis reaction (Scheme I, compound **1**) was demonstrated by incorporation of radioactive sulfite into the protein. Furthermore the new thiol group in **1** could be carboxymethylated to the disubstituted thioredoxin (compound **2**) whereas no reaction occurred between oxidized thioredoxin and iodoacetate prior to sulfite treatment. Therefore, sulfitolysis could also be conveniently followed using tritiated iodoacetate as a marker.

In either variant, product formation and separation from unreacted thioredoxin were readily detected on native polyacrylamide electrophoresis gels (Fig. 1). Thioredoxin-*S*-sulfonate **1** and carboxymethylthioredoxin-*S*-sulfonate **2** possess one or two additional negative charges and consequently migrate faster to the anode on non-denaturing gels. Autoradiography has confirmed the presence of radioactive sulfur in these products, but not in the starting material (Fig. 1, lane 6).

On a preparative scale, sulfitolysis reaction mixtures were freed from excess sulfite by gel filtration and the separation of unreacted and modified thioredoxins was achieved by DEAE cellulose chromatography (Fig. 2). However, because sulfitolysis is a reversible reaction and thioredoxin-*S*-sulfonate **1** is of limited stability (see below), **1** cannot be obtained as homogenous protein by conventional chromatography.

Optimum conditions for thioredoxin sulfitolysis were therefore established by measuring the formation of stable, [^{3}H]carboxymethylated derivative **2** after desalting of reaction mixtures. The reaction is only weakly affected by pH in the range between 6.5 and 8.5 but a slight maximum is observed at pH 8.0. Unfolding of thioredoxin by guanidinium chloride [20] increases sulfitolysis 3–6-fold. However, native thioredoxin is also substantially degraded by sulfite (cf. Fig. 1, lane 4) in contrast to other proteins which are resistant even towards high sulfite concentrations in the absence of denaturants. Using 5 mM sulfite concentration and a 500-fold molar excess over thioredoxin, up to 6% of the starting protein was modified within 2 h in buffer solution alone, and up to 20% in the presence of guanidinium chloride. Sulfitolysis was also detectable

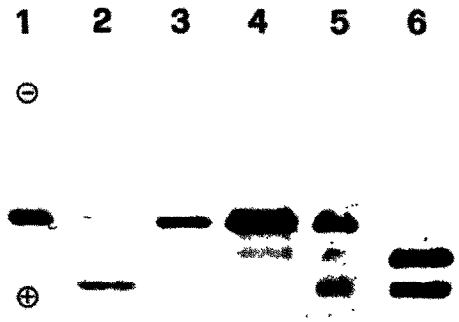


Fig. 1. Non-denaturing polyacrylamide gel electrophoresis of *E. coli* thioredoxin sulfitolysis products. (Lanes 1 and 3) Oxidized thioredoxin. (Lane 2) Di-*S*-carboxymethylthioredoxin; monocarboxymethylthioredoxin migrates to an intermediate position (not shown). (Lane 4) Sulfitolysis in buffer, without iodoacetate treatment (carboxymethylation), containing product **1**. (Lane 5) Sulfitolysis in 6 M guanidinium chloride followed by carboxymethylation (products **1** and **2**). (Lane 6) Autoradiogram of a sulfitolysis reaction with [^{35}S]sulfite, followed by carboxymethylation (^{35}S-labeled products **1** and **2**).

at still lower sulfite-to-thioredoxin ratios. Reaction periods longer than 4–6 h did not further increase the yield of thioredoxin-*S*-sulfonates under the conditions employed in this study.

The biochemical and physiological potential of primary product **1** of thioredoxin sulfitolysis depends on its rate of formation and upon its stability in solution: if the newly formed SH group in **1** is not trapped by added oxidants or alkylating agents, the thiolate can resubstitute the adjacent sulfur atom under elimination of sulfite. To determine the lifetime of **1**, sulfitolysis mixtures containing thioredoxin and [^{35}S]sulfite were rapidly desalted by FPLC and aliquots of the freshly prepared protein were stored at 0°C and 25°C for

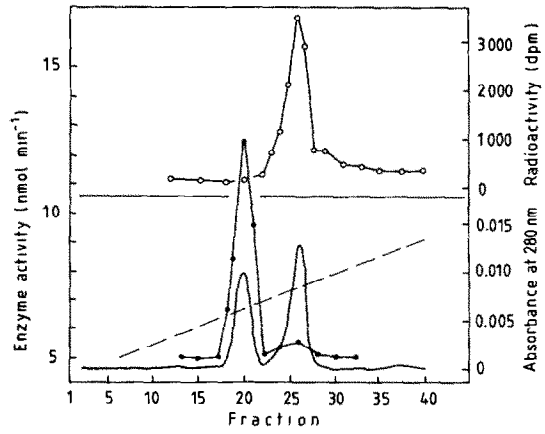


Fig. 2. DEAE cellulose chromatography of *E. coli* thioredoxin after sulfitolysis by [^{35}S]sulfite and carboxymethylation. Fraction 20 contains the unreacted thioredoxin and fraction 26 the disubstituted product **2**. (●) Stimulation of chloroplast NADP-MDH (left scale); (○) radioactivity (upper right scale); (——) protein elution profile (lower right scale); (– – –) 0–200 mM NaCl gradient.

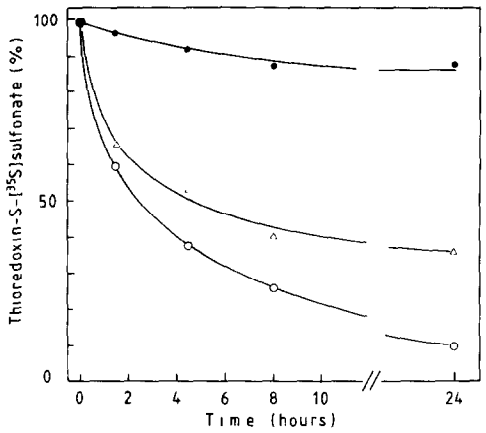


Fig. 3. Stability of ^{35}S-labeled thioredoxin-*S*-sulfonate derivatives **1** and **2** in solution at pH 8. (●) Carboxymethylated product **2**; (△) product **1** at 0°C; (○) product **1** at 25°C. The 100% value corresponds to a radioactivity of 900–1100 dpm per sample.

various periods of time. The protein was then rechromatographed by FPLC gel filtration, to remove any liberated sulfite, and the remaining radioactivity was determined in the protein. As shown in Fig. 3, little loss occurred in a control sample of radioactive **2** whereas the concentration of thioredoxin-*S*-sulfonate **1** had decreased to half the original amount within 3 h at 25°C. As expected, the disappearance of **1** was greatly enhanced by addition of 2 mM mercaptoethanol.

Finally we tested the capacity of modified thioredoxins **1** and **2** to activate a typical thioredoxin-dependent enzyme, viz. chloroplast NADP malate dehydrogenase. Purified preparations were unable to stimulate the enzyme even at high concentrations. In desalted reaction mixtures containing unreacted (about 90%) plus modified thioredoxins (about 10%), enzyme activation was less than half the expected value, indicating inhibition by the substituted protein. However, enzyme activities appeared to depend upon the proportion of active and inactive thioredoxin and upon reaction time in these experiments, necessitating much more comprehensive kinetic studies.

The above results provide an experimental basis for the hypothesis that sulfite inactivation of thioredoxins accounts for many of the inhibitory effects of sulfur dioxide in plants [21,22], in particular in the dark where the oxidized form predominates [9]. Sulfitolysis of chloroplast enzymes has been implicated in the phytotoxic action of SO_2 [23] but the common redox control system of thioredoxins appears a more likely target. Our reaction conditions approach reasonable physiological circumstances with respect to intracellular pH, thioredoxin concentrations [9], uptake and accumulation of sulfite in chloroplasts [24], and time scale in the range of hours. The use of *E. coli* thioredoxin, which is available in substrate quantities, as model protein is possible because of the high degree of sequence and conformational homology among thioredoxins in general, and because the bacterial protein can in vitro mimic any other thioredoxin in any enzyme system studied so far. An extension of the sulfitolysis experiments to different plant thioredoxins and to other enzymes of chloroplast metabolism is currently under way.

Acknowledgements: This study has been supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 305 (Ökophysiologie) and by Fonds der Chemischen Industrie.

REFERENCES

[1] Holmgren, A. (1985) Annu. Rev. Biochem. 54, 237–271.
[2] Buchanan, B.B., Wolosiuk, R.A. and Schürmann, P. (1979) Trends Biochem. Sci. 4, 93–96.
[3] Gleason, F.K. and Holmgren, A. (1988) FEMS Microbiol. Rev. 54, 271–298.
[4] Holmgren, A., Söderberg, B.O., Eklund, H. and Brändén, C.I. (1975) Proc. Natl. Acad. Sci. USA 72, 2305–2309.
[5] Wolosiuk, R.A., Crawford, N.A., Yee, B.C. and Buchanan, B.B. (1979) J. Biol. Chem. 254, 1627–1632.
[6] Schürmann, P., Maeda, K. and Tsugita, A. (1981) Eur. J. Biochem. 116, 37–45.
[7] Langlotz, P., Wagner, W. and Follmann, H. (1986) Z. Naturforsch. 41c, 275–283 and 979–987.
[8] Buchanan, B.B. (1980) Annu. Rev. Plant Physiol. 31, 341–374.
[9] Scheibe, R. (1981) FEBS Lett. 133, 301–304.
[10] Rebeille, F. and Hatch, M.D. (1986) Arch. Biochem. Biophys. 249, 171–179.
[11] Cecil, R. and McPhee, J.R. (1954) Biochemistry 60, 496–497.
[12] Bailey, J.L. and Cole, R.D. (1959) J. Biol. Chem. 234, 1733–1739.
[13] Thannhauser, T.W., Konishi, Y. and Scheraga, H.A. (1984) Anal. Biochem. 138, 181–188.
[14] Kella, N.K.D., Kang, Y.J. and Kinsella, J.E. (1988) J. Protein Chem. 7, 535–548.
[15] Kallis, G.B. and Holmgren, A. (1980) J. Biol. Chem. 255, 10261–10265.
[16] Jacquot, J.P., Vidal, P. and Schürmann, P. (1978) FEBS Lett. 96, 243–246.
[17] Willing, A., Follmann, H. and Auling, G. (1988) Eur. J. Biochem. 175, 167–173.
[18] Oakley, B.R., Kirsch, D.R. and Morris, N.R. (1980) Anal. Biochem. 105, 361–367.
[19] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[20] Kelley, R.F. and Stellwagen, E. (1984) Biochemistry 23, 5095–5102.
[21] Tanaka, K., Mitsuhashi, H., Kondo, N. and Sugahara, K. (1982) Plant Cell Physiol. 23, 1467–1470.
[22] Kropff, M.J. (1987) Plant Cell Environ. 10, 753–760.
[23] Lendzian, K.J. (1987) Naturwissenschaften 74, 282–288.
[24] Miszalski, Z. and Ziegler, H. (1989) Z. Naturforsch. 44c, 509–513.