FEBS Letters 455 (1999) 162–164 FEBS 22318

Redox control of hydrogenase activity in the green alga *Scenedesmus obliquus* by thioredoxin and other thiols

Röbbe Wünschiers[a,*], Heinrich Heide[b], Hartmut Follmann[b], Horst Senger[a], Rüdiger Schulz[a,c]

[a] *Fachbereich Biologie/Botanik, Philipps-Universität, Karl-von-Frisch-Str., D-35032 Marburg, Germany*
[b] *Fachbereich Biologie-Chemie, Biochemie, der Universität Kassel, D-34109 Kassel, Germany*
[c] *Botanisches Institut, Christian-Albrecht-Universität, Olshausenstr. 40, D-24098 Kiel, Germany*

Received 3 May 1999; received in revised form 15 June 1999

Abstract The activity of the NiFe-hydrogenase from the green alga *Scenedesmus obliquus* is inhibited by both algal thioredoxins f and I+II, and by *Escherichia coli* thioredoxin. The strongest inhibition was observed with homologous chloroplastic thioredoxin f (I_{50} = 21 nM) and *E. coli* thioredoxin (I_{50} = 83 nM). For the homologous cytoplasmic thioredoxins I+II an I_{50} of 667 nM was determined. Glutathione shows a similar but much less pronounced inhibitory effect whereas dithiothreitol had no effect. In addition to glucose-6-phosphate dehydrogenase, NiFe-hydrogenase is only the second enzyme known to be inhibited by reduced thioredoxin.

Key words: Enzyme regulation; Glutathione; Hydrogenase; Redox modulation; Thioredoxin; *Scenedesmus obliquus*

1. Introduction

Plant cells contain a multitude of at least four to six different thioredoxins engaged in the redox control of enzyme activities and other protein functions by reversible dicysteine-disulfide changes [1,2]. Light-dark regulation of several Calvin cycle enzymes mediated by the two chloroplast thioredoxins f and m is particularly well known [3]. Although more than 20 different such reactions have now been described in plants [2], the list is still incomplete, thioredoxin control of ammonia fixation in chloroplasts being the most recent addition [4]. Understanding the regulatory networks which link different metabolic pathways clearly requires further knowledge of potential thioredoxin targets. It is important in such studies in vitro to combine enzymes and thioredoxins from the same organism to avoid unspecific cross-reactivities frequently observed in heterologous systems.

We have previously characterized the thioredoxin profile of the unicellular green alga *Scenedesmus obliquus*, which possesses three thioredoxins of regular size (12 kDa) [5] and an unusually large chloroplast thioredoxin f (28 kDa) [6,7]. The latter is active in stimulating light-dependent algal NADP-malate dehydrogenase, fructose-bisphosphatase, and protochlorophyllide reductase [5,6,8], whereas the regular thioredoxins serve as hydrogen donors for algal ribonucleotide reductase [9].

In the present communication we present the effect of thioredoxin and other thiols on the enzyme responsible for H_2 metabolism in *S. obliquus* [10]. Under anaerobic conditions *S. obliquus* is capable of either light-dependent hydrogen production (photohydrogen production) or hydrogen uptake (photoreduction of CO_2) [11–13]. Both reactions are mediated by a hydrogenase which is situated within the chloroplast and has been purified and characterized as a NiFe enzyme [14,15]. We could recently show by Western blot investigations that this NiFe-hydrogenase is constitutively expressed. We thus propose a crucial role of the enzyme in algal photosynthesis. Conflicting reports of the effect of thiols on hydrogenase activity in bacteria [16,17,26] have prompted us to investigate the interaction of algal thioredoxins with the hydrogenase of the unicellular green algae. For the first time we here report an inactivation of an eukaryotic NiFe-hydrogenase by homologous thioredoxin in vitro.

2. Materials and methods

All chemicals and reagents were of the highest purity available. Antiserum raised against spinach chloroplast ferredoxin-thioredoxin reductase [18] was a generous gift of Professor P. Schürmann, University of Neuchatel, Switzerland. Protein was quantified by the method of Bradford [19], using bovine serum albumin as a standard. Protein molecular masses and purity were determined by 15% SDS-PAGE [20]. Proteins were visualized by Coomassie R-250 staining [21].

The thioredoxins of *S. obliquus* were partially purified and resolved into chloroplastic (f) and cytosolic (I, II) fractions by heat denaturation and gel filtration as described [5,6]. They were highly active in the standard fructose-bisphosphatase and NADP-malate dehydrogenase activation assays. Thioredoxin from *Escherichia coli* was purified from an overproducing strain by the published procedure [22].

All steps to partially purify the NiFe-hydrogenase of *S. obliquus* were carried out in an anaerobic chamber (Coylab, Grass Lake, MI, USA) under N_2 containing 10% H_2. A cell-free homogenate of anaerobically adapted cells was prepared as described [14], adjusted to an ammonium sulfate concentration of 30% (w/v) saturation, and centrifuged for 10 min at 48 000 × *g* and 4°C. The pellet was discarded and the supernatant adjusted to 50% (w/v) saturation in ammonium sulfate. After centrifugation the supernatant was discarded and the pellet resuspended in a basal buffer (50 mM Tris-HCl, pH 7.5) and desalted using Econo-Pac 10 DG desalting columns with 10 ml bed volume and a Bio-Gel P-6 matrix (Bio-Rad Laboratories, Hercules, CA, USA). Equilibration and elution were carried out with basal buffer. The desalted fractions were pooled and further purified by anion exchange chromatography. For all chromatographic steps 15% (v/v) glycerol was added to the basal buffer. The Q-Sepharose fast flow matrix (Sigma, Deisenhofen, Germany) was equilibrated with basal buffer; column dimensions were 11 × 3.5 cm and flow rate 3 ml/min. After application of the sample the matrix was washed with basal buffer and then with basal buffer containing 200 mM NaCl. Elution of the hydrogenase was performed by applying 4 column volumes of a linear NaCl gradient (200–500 mM) in basal buffer. The active fractions were collected and applied to a gel filtration column of Sephacryl S-200 (Pharmacia, Freiburg, Germany; dimensions: 66 × 2.6 cm) that had been previously equilibrated with basal buffer containing 100 mM NaCl. Elution was performed with the same buffer at a flow rate

*Corresponding author. Fax: (49) (6421) 282057.
E-mail: senger@mailer.uni-marburg.de

PII: S0014-5793(99)00867-4

of 1 ml/min. The active hydrogenase fractions were used for the experiments.

Hydrogenase activity was assayed spectroscopically as H_2 uptake rate in gas-tight 1 ml cuvettes under anaerobic conditions. For testing the influence of thiols on hydrogenase activity 1 U/ml hydrogenase was incubated with changing concentrations of thiols for 10 min. Thioredoxin was reduced by addition of 1 mM dithiothreitol. The H_2 uptake reaction was started by adding 10 mM oxidized methylviologen. All chemicals were dissolved in basal buffer supplemented with 15% (v/v) glycerol and equilibrated with 10% H_2 in N_2. Rates of H_2 oxidation were measured by following the reduction of methylviologen at 578 nm ($\varepsilon_{578} = 9700$ cm^2/mmol).

3. Results and discussion

Bacterial NiFe-hydrogenases possess a large number of cysteine residues not all of which appear to be engaged in the formation of FeS clusters and the nickel- and iron-containing active site [23,24]. The enzymes are thus likely candidates for dithiol/disulfide redox changes and activity modulation. The idea of thioredoxin as regulator for bacterial hydrogenases was first suggested by Spiller et al. [25]. Fernandez et al. [26] observed no effect of thioredoxin m from spinach and thioredoxin from *E. coli* on the periplasmic NiFe-hydrogenase from *Desulfovibrio gigas*. However, Papen et al. [16] reported an activation of the uptake (but not of the reversible) NiFe-hydrogenase from the cyanobacterium *Anabaena* PCC 7119 with homologous thioredoxin m. To resolve the dependence of hydrogenase activity in *S. obliquus* on thiols, the algal enzyme was preincubated with varying amounts of thiols for 10 min and H_2 uptake activity was then tested photometrically by starting the reaction with methylviologen as electron acceptor. We have assayed the algal hydrogenase in the presence of dithiothreitol, glutathione, and of the most likely physiological reductant thioredoxin. Both the common model protein, thioredoxin from *E. coli*, and homologous thioredoxins isolated from *S. obliquus* were used.

A clear indication for redox modulation of *S. obliquus* NiFe-hydrogenase was obtained with reduced *E. coli* thioredoxin which effectively inhibits hydrogenase activity (Fig. 1).

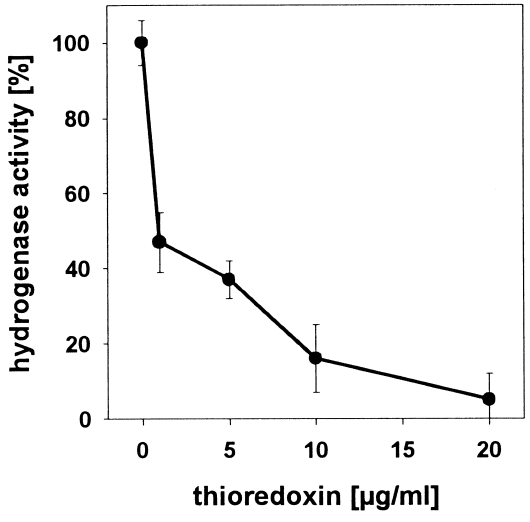

Fig. 1. Activity of the NiFe-hydrogenase from *S. obliquus* with various concentrations of *E. coli* thioredoxin in vitro. The hydrogenase was preincubated with 1 mM dithiothreitol and thioredoxin for 10 min. H_2 oxidation was started by addition of 10 mM oxidized methylviologen as an artificial electron acceptor. 100% activity corresponds to the oxidation of 10 μmol H_2/min × ml.

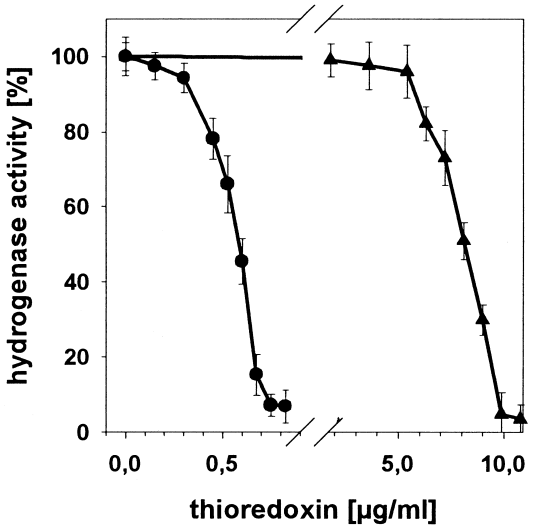

Fig. 2. Activity of the NiFe-hydrogenase from *S. obliquus* under various concentrations of homologous chloroplastic thioredoxin f (●) and cytosolic thioredoxin I+II (▲) in vitro. Parameters as in Fig. 1.

50% inhibition (I_{50}) was achieved in the presence of about 83 nM (1 μg/ml) thioredoxin. Likewise chloroplastic thioredoxin f and cytoplasmic thioredoxin I+II from *S. obliquus* exhibit strong inhibitory effects (Fig. 2). I_{50} values were estimated to be 21 nM (0.6 μg/ml) and 667 nM (8 μg/ml), respectively. Reduced glutathione also inhibits algal hydrogenase activity albeit at more than 10^4-fold concentrations (Fig. 3) whereas dithiothreitol does not at all affect the enzyme in vitro. No inhibition was observed with bovine serum albumin instead of thioredoxin. Surprisingly, some inhibition of hydrogenase activity was also observed in control experiments carried out in the presence of oxidized thioredoxin and glutathione (cf. Fig. 3). We suppose that these oxidized species are slowly reduced, enzymatically or non-enzymatically, under the reducing in vitro assay conditions. By Western blot investigations with antiserum raised against spinach chloroplast ferredoxin-thioredoxin reductase [18] we tested all thioredoxin and hydrogenase preparations. However, no cross-reaction was observed in any case, making the interference from ferredoxin-thioredoxin reductase unlikely.

These results show the existence of a thioredoxin-accessible site at the hydrogenase protein. Although neither the sequence nor the structure of the chloroplast hydrogenase from *S. obliquus* is as yet known, we suppose it to be homologous to the bacterial enzymes since these hydrogenases are highly conserved [23]. As a model we checked the two known three-dimensional structures of bacterial NiFe-hydrogenases from *Desulfovibrio gigas* [24] and *D. fructosovorans* [27] for potential thioredoxin targets by computer analysis using Swiss-Pdb Viewer [28]. Since there are no consensus sequences in thioredoxin-regulated enzymes [2], only the crystal structure can show up surface-exposed cysteines. The active site-containing large subunit from *D. fructosovorans* indeed contains two surface-exposed cysteine residues (Cys-259 and Cys-436) [27,29] which are not involved in the coordination of any FeS cluster and thus are good candidates for an interaction with thioredoxin. Their sulfur atoms are at a distance of 2.56 Å from each other. For comparison, the two redox-active sulfurs in *E. coli* thioredoxin have a distance of 2.01 Å and 3.82 Å in the

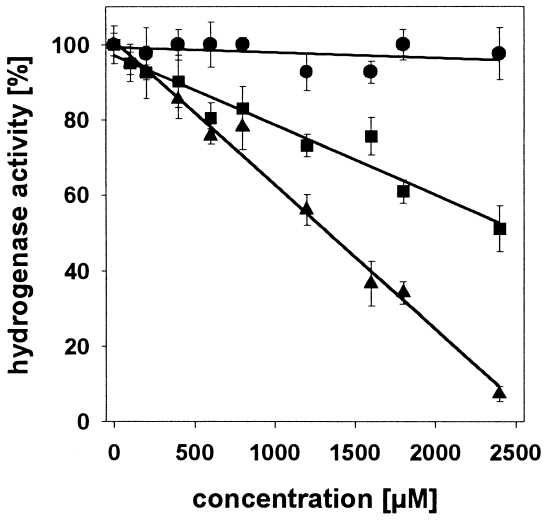

Fig. 3. Different effect of oxidized (■) and reduced (▲) glutathione and dithiothreitol (●) on hydrogenase activity in *S. obliquus* in vitro. Other parameters as in Fig. 1.

oxidized and reduced state, respectively [30]. In contrast, no nearby surface-exposed cysteines exist in the NiFe-hydrogenase from *D. gigas* [24], in accord with the fact that no thioredoxin-mediated activity modulation was observed for this enzyme [26].

Our results clearly show redox regulation of the NiFe-hydrogenase from *S. obliquus* by thiols. The effect is highly specific for the reduced homologous chloroplast thioredoxin f of the alga (Fig. 2) but can be mimicked by other thioredoxins in vitro. The lack of a dithiothreitol effect and poor efficiency of reduced glutathione indicate that the hydrogenase has to engage in distinct protein-protein interactions with thioredoxin for activity modulation, as has been observed for many thioredoxin-regulated chloroplast enzymes [2,31]. Such specific interactions also explain the differences in the concentration saturation curves observed between heterologous (Fig. 1) and homologous (Fig. 2) protein combinations or glutathione, respectively (Fig. 3).

The specific, negative hydrogenase modulation by thioredoxin observed in vitro provides a physiological mode of regulating H_2 and CO_2 metabolism in the algae [32,33]. Since the hydrogenase drains electrons from photosystem I via ferredoxin (to be published elsewhere) and thus competes for electrons with the Calvin cycle, an inhibition of hydrogenase activity parallel to the activation of Calvin cycle enzymes by thioredoxin during oxygenic photosynthesis can indeed be expected. Next to inhibition of plastid and cyanobacterial glucose-6-phosphate dehydrogenase by reduced thioredoxins [3] this is only the second case of negative thioredoxin control. It will also be interesting to evaluate the interaction of other microbial hydrogenases, especially the enzyme from *D. fructosovorans*, with their homologous cellular thioredoxins.

Acknowledgements: These studies have been supported by Deutsche Forschungsgemeinschaft, COST Action 818 of the European Union and by a scholarship to R.W. from the Studienstiftung des Deutschen Volkes.

References

[1] Buchanan, B.B. (1980) Annu. Rev. Plant Physiol. 31, 341–374.
[2] Follmann, H. and Häberlein, I. (1996) BioFactors 5, 147–156.
[3] Jacquot, J.-P. and Lancelin, J.-M. (1997) New Phytol. 136, 543–570.
[4] Lichter, A. and Häberlein, I. (1998) J. Plant Physiol. 153, 83–90.
[5] Langlotz, P., Wagner, W. and Follmann, H. (1986) Z. Naturforsch. 41c, 979–987.
[6] Langlotz, P., Wagner, W. and Follmann, H. (1986) Z. Naturforsch. 41c, 275–283.
[7] Langlotz, P. and Follmann, H. (1987) Z. Naturforsch. 42c, 1364–1366.
[8] Kotzabasis, K., Senger, H., Langlotz, P. and Follmann, H. (1989) J. Photochem. Photobiol. 3, 333–339.
[9] Hofmann, R., Feller, W., Pries, M. and Follmann, H. (1985) Biochim. Biophys. Acta 832, 98–112.
[10] Appel, J. and Schulz, R. (1998) J. Photochem. Photobiol. 47, 1–11.
[11] Gaffron, H. (1939) Nature 143, 204–205.
[12] Gaffron, H. (1940) Am. J. Bot. 27, 273–283.
[13] Gaffron, H. and Rubin, J. (1942) J. Gen. Physiol. 26, 219–240.
[14] Schnackenberg, J., Schulz, R. and Senger, H. (1993) FEBS Lett. 327, 21–24.
[15] Zinn, T., Schnackenberg, J., Haak, D., Römer, S., Schulz, R. and Senger, H. (1994) Z. Naturforsch. 49c, 33–38.
[16] Papen, H., Kentemich, T., Schmulling, T. and Bothe, H. (1986) Biochimie 68, 121–132.
[17] Fagan, T.F. and Mayhew, S.G. (1993) Biochem. J. 293, 237–241.
[18] Schürmann, P. (1995) Methods Enzymol. 252, 274–283.
[19] Bradford, M.M. (1979) Anal. Biochem. 72, 248–254.
[20] Fling, S.P. and Gregerson, D.S. (1986) Anal. Biochem. 155, 93–98.
[21] Chua, N.-H. and Bennoun, P. (1975) Proc. Natl. Acad. Sci. USA 72, 2175–2179.
[22] Langsetmo, K., Fuchs, J. and Woodward, C. (1989) Biochemistry 28, 3211–3220.
[23] Wu, L.F. and Mandrand, M.A. (1993) FEMS Microbiol. Rev. 10, 243–269.
[24] Volbeda, A., Charon, M.H., Piras, C., Hatchikian, E.C., Frey, M. and Fontecilla-Camps, J.C. (1995) Nature 373, 580–587.
[25] Spiller, H., Bookjans, G. and Shanmugam, K.T. (1983) J. Bacteriol. 155, 129–137.
[26] Fernandez, V.M., Hatchikian, E.C. and Cammack, R. (1985) Biochim. Biophys. Acta 832, 69–79.
[27] Montet, Y., Amara, P., Volbeda, A., Vernede, X., Hatchikian, E.C., Field, M.J., Frey, M. and Fontecilla-Camps, J.C. (1997) Nature Struct. Biol. 4, 523–526.
[28] Guex, N. and Peitsch, M.C. (1997) Electrophoresis 18, 2714–2723.
[29] Rousset, M., Dermoun, Z., Hatchikian, C.E. and Belaich, J.P. (1990) Gene 94, 95–101.
[30] Jeng, M.F., Campbell, A.P., Begley, T., Holmgren, A., Case, D.A., Wright, P.E. and Dyson, H.J. (1994) Structure 2, 853–868.
[31] Braun, H., Lichter, A. and Häberlein, I. (1996) Eur. J. Biochem. 240, 781–788.
[32] Boichenko, V.A. and Hoffmann, P. (1994) Photosynthetica 30, 527–552.
[33] Schulz, R. (1996) J. Mar. Biotechnol. 4, 16–22.