Metal-catalyzed inactivation of bovine glucose-6-phosphate dehydrogenase – role of thiols

Konrad L. Maier*, Helga Hinze, Barbara Meyer, Anke-Gabriele Lenz

GSF – National Research Center for Environment and Health, Institute of Inhalation Biology, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

Received 16 August 1996; revised version received 10 September 1996

Abstract The role of thiols as oxidant scavengers during inactivation of bovine glucose-6-phosphate dehydrogenase by metal-catalyzed oxidation systems has been studied in vitro. Partial inactivation of the enzyme was achieved by the metal-catalyzed oxidation systems Fe(II)/H_2O_2/EDTA or Fe(II)/H_2O_2/ADP under specific conditions. When EDTA as chelator was present in the oxidation system, both cysteine and *N*-acetylcysteine at low concentrations (0.1–1 mM) drastically enhanced inactivation, while cysteinyl-glycine and glutathione did not. The thiol-mediated inactivation was inhibitable by superoxide dismutase. Depletion of enzyme activity by cysteine was paralleled by an increase of the carbonyl content, which indicates oxidative injury. However, when EDTA as chelator was replaced by the natural chelator ADP, all thiols studied acted as antioxidants. It is therefore concluded that the nature of the chelator as a constituent of the metal-catalyzed oxidation systems determines whether the antioxidative function of some thiols is shifted to a prooxidative function against glucose-6-phosphate dehydrogenase.

Key words: Antioxidant; Cysteine; Glutathione; Metal-catalyzed oxidation; *N*-Acetylcysteine; Thiol

1. Introduction

Thiols are efficient scavengers of reactive oxygen metabolites, such as hydrogen peroxide, chlorinated amines or hydroxyl radicals [1]. The major thiol-containing substance playing an essential role in antioxidant defence mechanisms at inflammatory sites is glutathione. Its concentration in the lung epithelial lining fluid of normal nonsmokers amounts to about 0.4 mM [2]. Subnormal levels of GSH are found in chronic inflammations such as idiopathic pulmonary fibrosis [3,4] or cystic fibrosis [5], which are characterized by an increased oxidant burden [6,7]. Pulmonary glutathione deficiency represents a severe risk for lung tissue injury. Systemic application of *N*-acetylcysteine (NAC) is one promising approach to support directly the antioxidant capacity or to replenish GSH levels. This approach is currently evaluated for treatment of inflammatory lung diseases with the feature of ever GSH-deficiency [8,9].

In addition to their protective function against toxic oxygen radicals, thiols have been shown to enhance DNA oxidation [10] and lipid peroxidation [11], catalyzed by transition metal ions via a thiyl radical (R-S$^{\bullet}$)-dependent mechanism. At physiological conditions, free Fe(II) or Fe(III) ions occur at extremely low concentrations in tissues or organs, since they are bound to ferritin, transferrin or lactoferrin, thus being not available for metal-catalyzed oxidation (MCO) systems. However, at inflamed sites, iron ions might be mobilized from the stores by oxidative [12–15] or proteolytic mechanisms [16], thus increasing the risk of oxidative damage by hydroxyl radicals generated via Fenton-like systems. In context with the therapeutic administration of thiols, e.g. in inflammatory lung diseases showing strong GSH depletion, we asked the question whether thiols at local conditions might enhance oxidative damage of relevant enzymes or proteins by MCO systems, instead of protecting them from reactive oxygen metabolites. In an in vitro approach, we studied the effect of several thiols on the metal-catalyzed oxidation of mammalian glucose-6-phosphate dehydrogenase (GPDH), a target enzyme being sensitive to oxidative stress [17,18]. We found that both cysteine (CYS) and NAC drastically enhanced inactivation of the enzyme by an EDTA-dependent MCO system, while GSH and cysteinyl-glycine did not.

2. Materials and methods

2.1. Materials

GPDH from bovine adrenals (4.6 mg/ml) was from Sigma, Deisenhofen. Stock solutions of GPDH used for the incubation experiments were obtained by 1:10 dilution of the enzyme suspension with 20 mM potassium phosphate buffer, pH 7.4. Xanthine oxidase (XOD) from cow milk (175 mU/mg lyophilisate), superoxide dismutase (SOD) from bovine erythrocytes (5000 U/mg) and catalase (CAT) from bovine liver (65 000 U/mg) were from Boehringer, Mannheim. All other chemicals were from Merck, Darmstadt.

2.2. Inactivation of GPDH by metal-catalyzed oxidation

Inactivation studies were performed with 0.24 mg/ml GPDH in 20 mM potassium phosphate buffer, pH 7.4, in a total volume of 0.125 ml, using two different MCO systems which are specified below. The incubations at 37°C were terminated after 30 min by cooling the reaction mixtures on ice. Enzyme activity was measured according to [19]. The EDTA-dependent MCO system consisted of 0.9 mM Fe(II), 3.3 mM H_2O_2 and 0.05 mM EDTA resulting in 30–50% inactivation of GPDH under standard conditions. In the ADP-dependent MCO system, EDTA was replaced by 2 mM ADP, resulting in 50–60% inactivation of the enzyme under standard conditions. To study the influence of thiols on basal inactivation by the MCO systems, the reaction mixtures additionally contained 0.1, 0.2, 1.0 or 5.0 mM CYS, NAC, cysteinyl-glycine or GSH. The Fe(II)/H_2O_2/EDTA-dependent incubations being completed by CYS were carried out in the absence and presence of SOD (20 U/ml or 200 U/ml) or BSA (4 μg/ml or 40 μg/ml). BSA was considered an unspecific radical scavenger protein. Incubations were made in triplicate, if not otherwise stated in Tables 1 and 3.

2.3. Inactivation of GPDH by xanthine oxidase/hypoxanthine

Inactivations were performed in 0.1 M potassium phosphate buffer, pH 7.6, using 0.24 mg/ml GPDH, 6.3 mU/ml XOD, 0.3 mM hypoxanthine in the absence and presence of SOD (50 U/ml) or CAT (650

*Corresponding author. Fax: (49) (89) 3187-2400.

Abbreviations: BSA, bovine serum albumin; CAT, catalase; CYS, cysteine; GPDH, glucose-6-phosphate dehydrogenase; GSH, glutathione; MCO, metal-catalyzed oxidation; NAC, *N*-acetylcysteine; SOD, superoxide dismutase

PII S0014-5793(96)01054-X

U/ml) in a total volume of 0.125 ml. Incubations were carried out for 30 min at 37°C and then stopped by cooling the reaction mixtures on ice [20].

2.4. Analysis of oxidized GPDH for carbonyl content

Separate incubations with GPDH were performed using the EDTA-dependent MCO system to study the influence of CYS (0.1, 0.2 and 5 mM) on formation of protein carbonyls in parallel to inactivation. Conditions of incubation were identical to that described in Section 2.2. Analysis of protein carbonyl groups has been described elsewhere [21,22]. All incubations were made in triplicate.

3. Results

3.1. Effect of thiols on GPDH activity in the presence of Fe(II), Fe(II)/EDTA, Fe(II)/H_2O_2 or Fe(II)/H_2O_2/EDTA

Enzyme activity after treatment with 0.9 mM Fe(II) in the absence of both EDTA and H_2O_2 under standard conditions amounted to 84.7% of control activity. The remaining activity after incubation in the presence of Fe(II)/EDTA (0.9/0.05 mM) or Fe(II)/H_2O_2 (0.9/3.3 mM) was 82% or 56%, respectively. The presence of CYS in both systems did not markedly modulate inactivation (Table 1). Similar results were found for NAC and GSH (data not shown).

Treatment of the enzyme with the complete EDTA-dependent MCO system (Fe(II)/H_2O_2/EDTA (0.9/3.3/0.05 mM)) resulted in a similar inactivation as treatment in the absence of EDTA. However, the presence of low concentrations (0.1–1 mM) of CYS or NAC in this system drastically enhanced inactivation of GPDH, while higher thiol concentrations (5 mM) were less or not efficient. The additional inactivation of GPDH by CYS was prevented by SOD. In contrast to CYS or NAC, the presence of cysteinyl-glycine or GSH did not promote inactivation by Fe(II)/H_2O_2/EDTA (Table 1).

Table 1
Modulation of Fe(II)/H_2O_2-catalyzed inactivation of GDPH by thiols in the presence of EDTA

Addition	Activity in % of control (mean ± S.D.)
None (control)	100[a]
Fe(II)	84.7 ± 11
Fe(II)/EDTA	82.0 ± 18.5
+0.1 mM CYS	77.7 ± 1.50
+1.0 mM CYS	77.3 ± 5.50
+5.0 mM CYS	74.7 ± 13.6
Fe(II)/H_2O_2	56.0 ± 7.5
+0.2 mM CYS	70.0 ± 3.1
+1.0 mM CYS	57.7 ± 12.2
+2.0 mM CYS	70.3 ± 8.6
Fe(II)/H_2O_2/EDTA (n = 12)	54.3 ± 8.8
+0.1 mM CYS	14.0 ± 2.0
+0.2 mM CYS	4.5 ± 1.0
+1.0 mM CYS	4.7 ± 1.2
+5.0 mM CYS	49.3 ± 5.8
+1.0 mM CYS +4.0 μg SOD	13.0 ± 1.10
+1.0 mM CYS +40 μg SOD	75.6 ± 13.9
+1.0 mM CYS +4.0 μg BSA	5.0 ± 1.9
+1.0 mM CYS +40 μg BSA	8.5 ± 2.1
+0.1 mM NAC	7.3 ± 0.60
+0.2 mM NAC	3.9 ± 1.2
+1.0 mM NAC	5.6 ± 3.3
+5.0 mM NAC	51.5 ± 21.9
+0.2 mM GSH	46.0 ± 8.5
+0.5 mM GSH	37.0 ± 4.0
+5.0 mM GSH	76.0 ± 0
+0.1 mM CYS-GLY	63.5 ± 0.70
+1.0 mM CYS-GLY	62.7 ± 7.1
+5.0 mM CYS-GLY	58.3 ± 13.2

[a]The activity in the control was 20 ± 4 U/ml (mean ± S.D.).

3.2. Carbonyl content of GPDH after treatment with the EDTA-dependent MCO system

Untreated GPDH contained 0.16 mol carbonyl/mol enzyme subunit. Incubation with the EDTA-dependent MCO system increased the carbonyl level to 0.30 mol/mol enzyme subunit. A significant carbonyl formation being paralleled by substantial inactivation was found in the presence of 0.1 or 0.2 mM CYS. However, the highest CYS concentration (5 mM) caused less carbonyl formation, which was consistent with the lower rate of inactivation (Table 2). The increase of carbonyl content from 0.3 to 1.08 mol/mol enzyme subunit by 0.2 mM CYS was related to 65% loss of activity, suggesting a rather selective modification reaction at susceptible sites.

3.3. Effect of CYS on inactivation of G6PDH by Fe(II)/H_2O_2/ADP (ADP-dependent MCO system)

Treatment of the enzyme in the presence of Fe(II)/H_2O_2 (0.9/3.3 mM) and increasing concentrations of ADP as chelator (up to 2 mM) resulted in an further loss of activity (data not shown). Using 2 mM ADP in the incubations, inactivation of GPDH was substantially prevented by CYS, NAC or GSH (0.1–5 mM), thus demonstrating a clear scavenger function of thiols in the ADP-dependent MCO system (Table 3).

3.4. Effect of xanthine oxidase/hypoxanthine on GPDH activity

Treatment of GPDH with the xanthine oxidase/hypoxanthine system caused a substantial inactivation. Depletion of enzyme activity was prevented by SOD but not by CAT (Table 4), suggesting that superoxide ($O_2^{\bullet -}$) is the inactivating agent.

4. Discussion

Besides the role as the first enzyme in the pentose phosphate pathway, mammalian GPDH has a key function in phagocytes by providing NADPH equivalents for the respiratory burst oxidase. As recently reported, GPDH is sensitive to oxidants arising from metal-driven oxidation systems [17,18].

Table 2
Relationship between formation of carbonyl groups and inactivation of GPDH by Fe(II)/H_2O_2/EDTA in the presence of CYS

Addition	Carbonyl content in mol/mol enzyme subunit (mean ± S.D.)	Activity in % of control (mean ± S.D.)
None (control)	0.16 ± 0.03	100[a]
Fe(II)/H_2O_2/EDTA	0.30 ± 0.12	72.0 ± 10.0
+0.1 mM CYS	0.78 ± 0.06	20.0 ± 4.0
+0.2 mM CYS	1.08 ± 0.03	7.0 ± 4.5
+5.0 mM CYS	0.45 ± 0.03	64.0 ± 9.0

[a]For GPDH activity in the control, see Table 1.

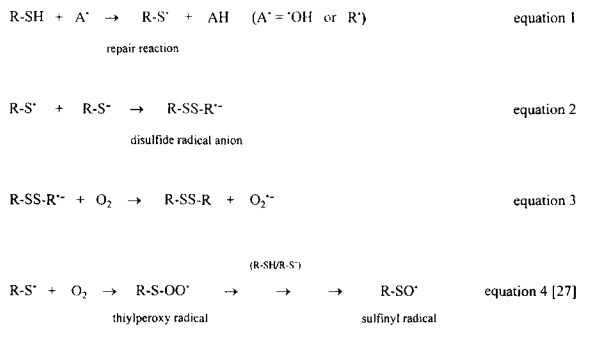
Table 3
Modulation of Fe(II)/H_2O_2-catalyzed inactivation of GDPH by CYS, NAC and GSH in the presence of ADP

Addition	Activity in % of control (mean ± S.D.)
None (control)	100[a]
Fe(II)/H_2O_2/ADP ($n=9$)	41.3 ± 7.2
+0.2 mM CYS	50.3 ± 9.0
+5.0 mM CYS	87.0 ± 4.2
+0.2 mM NAC	74.0 ± 0.0
+5.0 mM NAC	105.0 ± 4.0
+0.2 mM GSH	74.0 ± 9.9
+5.0 mM GSH	97.0 ± 25.0

[a] For GPDH activity in the control, see Table 1.

Oxidative inactivation of this enzyme might therefore be a regulatory mechanism to control reactive oxygen metabolite production by phagocytes at acute inflammatory reactions. In contrast to GPDH from *Leuconostoc mesenteroides* [23,24], the mammalian enzyme contains several cysteine residues [25]. Sulfhydryl groups, however, seem not to be essential for the catalytic activity of the mammalian enzyme as shown by thiol-modifying agents like iodoacetic acid (unpublished data). This observation is consistent with data recently published, showing that sulfhydryl oxidation has little effects on metal-catalyzed inactivation of mammalian GPDH [26]. Remarkably, the enzyme from *Leuconostoc mesenteroides* is highly susceptible to MCO systems in spite of the absence of cysteine residues [24].

In this study, we demonstrated that the presence of low concentrations of CYS or NAC drastically enhanced inactivation by the EDTA-dependent MCO system. This effect was prevented by SOD (Table 1), suggesting that superoxide ($O_2^{\bullet-}$) might play a role in the inactivation process. Superoxide anion generation is thought to result from formation of thiyl radicals (R-S$^\bullet$) which are released from the so-called repair reaction (Eq. 1). Thiyl radicals react with thiolates (R-S$^-$)to give disulfide radical anions (R-SS-R$^{\bullet-}$; Eq. 2), which are very efficient in reducing O_2 to $O_2^{\bullet-}$ (Eq. 3), thus increasing oxidant burden.



An alternative mechanism for inactivation is based on the reactivity of thiylperoxy (R-S-OO$^\bullet$) or sulfinyl radicals (R-SO$^\bullet$), which might be generated from R-S$^\bullet$ (Eq. 4). R-SO$^\bullet$ radicals have been proposed recently to be potent oxidants by abstracting hydrogen atoms from target molecules, while R-S$^\bullet$ radicals are less potent in causing oxidative damage [27]. Assuming that this alternative pathway is responsible for thiol-mediated inactivation of mammalian GPDH, prevention of inactivation by SOD could be explained by the possibility that R-S$^{\bullet-}$ radicals are withdrawn from the system via formation of R-SS-R$^{\bullet-}$ radicals (Eq. 2) followed by reduction of O_2 to $O_2^{\bullet-}$ (Eq. 3). In this case, $O_2^{\bullet-}$ would not be involved in inactivation of GPDH, but would represent a sink for toxic radicals as proposed by Winterbourn [28].

Table 4
Effect of xanthine oxidase/hypoxanthine (XOD) on GPDH activity

Addition	Activity in % of control (mean ± S.D.)
None (control)	100[a]
XOD ($n=8$)	53.8 ± 14.3
+SOD ($n=8$)	95.5 ± 12.5
+CAT ($n=7$)	61.7 ± 9.1

[a] For GPDH activity in the control, see Table 1.

We have provided evidence that treatment of GPDH with the XOD/hypoxanthine system inactivates GPDH. This inactivation is prevented by SOD but not by catalase (Table 4). Müller et al. [29] recently reported inactivation of mammalian GPDH by $O_2^{\bullet-}$, which is supported by our findings. On the basis of these data, we suppose that the CYS-mediated and SOD-inhibitable inactivation of GPDH during treatment with the EDTA-dependent MCO system is effected by $O_2^{\bullet-}$ and not by R-S-OO$^\bullet$ or R-SO$^\bullet$ radicals.

Analysis of protein carbonyls as an indicator of oxidative modification revealed that both the basal inactivation by the EDTA-dependent MCO system and the additional inactivation by CYS contribute to formation of carbonyl groups in mammalian GPDH. The increase of the carbonyl content to the maximum level of 1.08 mol/mol enzyme subunit is accompanied by 93% loss of activity, suggesting a site-specific oxidative modification which is supported by CYS (Table 3). A specific amino acid residue in the active site is postulated to be susceptible to oxidation. Szweda and Stadtman [24] recently found that oxidative inactivation of the bacterial enzyme by MCO systems was correlated with the formation of one carbonyl functionality/enzyme subunit, indicating a site-specific modification.

Kim et al. [30] described nonenzymatic cleavage of glutamine synthetase, another kind of oxidative modification, by catalytic amounts of iron salts in the presence of 10 mM dithiothreitol. This rather high concentration of thiol apparently recycles Fe(III) to Fe(II) which then reduces O_2 to $O_2^{\bullet-}$. We have no evidence whether exactly this is relevant in our EDTA-dependent inactivation system, which works with 100-fold lower concentrations of CYS or NAC. On the other hand, we cannot exclude that traces of CYS or NAC specifically influence the FE(II)/Fe(III)-redox system, e.g. by formation of iron-chelate complexes, which might enhance the kinetics of oxygen reduction to superoxide by the redox system.

In comparison to the EDTA-dependent MCO system, the ADP-dependent oxidation system was different in the response of thiols. ADP and ATP are natural chelators which support MCO systems [31,32]. In the ADP-driven system, GSH as well as CYS and NAC at low and high concentrations clearly acted as antioxidants (Table 2). We therefore conclude that the nature of the chelator as part of the MCO system determines whether a thiol behaves like an antioxidant or a prooxidant with regard to GPDH as target. So far, there is no evidence for the existence of natural chelators, which trigger thiols to act as prooxidants against GPDH. However, preliminary results on the inactivation of alkaline phosphatase by Fe(II) in the absence of both H_2O_2 and chelator indicate a prooxidative function of GSH, NAC and

CYS. In this case, we assume that the target protein itself may be part of the oxidation system, possibly by exhibiting a chelator-like property at the site being attacked.

In summary, we have shown for the first time that the presence of low concentrations of CYS or NAC in a selected MCO system mediates oxidative damage to the target protein, which is reflected in the introduction of carbonyl groups into the target protein and which causes a concomitant loss of biological function. Whether related mechanisms might be of relevance during antioxidant therapy in treatment of severe inflammatory diseases by thiols has to be clarified.

Acknowledgements: The authors thank Dr. K. Buff for critical reading of the manuscript.

References

[1] Aruoma, O.I., Halliwell, B., Hoey, B.M. and Butler, J. (1989) Free Radical Biol. Med. 6, 593–597.
[2] Cantin, A.M., North, S.L., Hubbard, R.C. and Crystal, R.G. (1987) J. Appl. Physiol. 63, 152–157.
[3] Cantin, A.M., Hubbard, R.C. and Crystal, R.G. (1989) Am. Rev. Respir. Dis. 139, 370–372.
[4] Cantin, A.M. and Bégin, R. (1991) Lung 169, 123–138.
[5] Roum, J.H., Buhl, R., McElvaney, N.G., Borok, Z. and Crystal, R.G. (1993) J. Appl. Physiol. 75, 2419–2424.
[6] Maier, K., Leuschel, L. and Costabel, U. (1991) Am. Rev. Respir. Dis. 143, 271–274.
[7] Behr, J., Maier, K., Krombach, F. and Adelmann-Grill, B.C. (1991) Am. Rev. Respir. Dis. 144, 146–150.
[8] Bridgeman, M.M.E., Marsden, M., MacNee, W., Flenley, D.C. and Ryle, A.P. (1991) Thorax 46, 39–42.
[9] Meyer, A., Buhl, R., Kampf, S. and Magnussen, H. (1995) Am. J. Respir. Crit. Care Med. 152, 1055–1060.
[10] Spear, N. and Aust, S.D. (1995) Arch. Biochem. Biophys. 317, 142–148.
[11] Schöneich, C., Dillinger, U., von Bruchhausen, F. and Asmus, K.D. (1992) Arch. Biochem. Biophys. 292, 456–467.
[12] Williams, D.M., Lee, G.R. and Cartwright, G.E. (1974) J. Clin. Invest. 53, 665–667.
[13] Harris, L.R., Cake, M.H. and Macey, D.J. (1994) Biochem. J. 301, 385–389.
[14] Thomas, C.E., Morehouse, L.A. and Aust, S.D. (1985) J. Biol. Chem. 260, 3275–3280.
[15] Gutterigde, J.M.C. (1986) FEBS Lett. 201, 291–295.
[16] Britigan, B.E. and Edeker, B.L. (1991) J. Clin. Invest. 88, 1092–1102.
[17] Maier, K., Hinze, H. and Holzer, H. (1991) Biochim. Biophys. Acta, 1079, 238–241.
[18] Oliver, C.N., Ahn, B.-W., Moermann, E.J., Goldstein, S. and Stadtman, E.R. (1987) J. Biol. Chem. 262, 5488–5491.
[19] Löhr, G.W. and Waller, H.D. (1974) in: Methoden der enzymatischen Analyse (Bergmeyer, H.U., Ed.), Vol. I, pp. 673–681, Verlag Chemie, Weinheim.
[20] Bray, R.C. (1975) in: The Enzymes, Vol. II, Oxidation-Reduction Part B (Boyer, P.D., Ed.), Academic Press, New York.
[21] Lenz, A.-G., Costabel, U., Shaltiel, S. and Levine, R.L. (1988) Anal. Biochem. 177, 419–425.
[22] Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.-G., Ahn, B.-W., Shaltiel, S. and Stadtman, E.R. (1990) Methods Enzymol. 186, 464–478.
[23] Ishaque, A., Milhausen, M. and Levy, H.R. (1974) Biochem. Biophys. Res. Commun. 59, 894–901.
[24] Szweda, L.I. and Stadtman, E.R. (1992) J. Biol. Chem. 267, 3096–3100.
[25] Takizawa, T., Huang, I.-Y., Ikuta, T. and Yoshida, A. (1986) Proc. Natl. Acad. Sci. USA 83, 4157–4161.
[26] Ogina, T. and Okada, S. (1995) Biochim. Biophys. Acta 1245, 359–365.
[27] Yim, M.B., Chae, H.Z., Rhee, S.G., Chock, P.B. and Stadtman, E.R. (1994) J. Biol. Chem. 269, 1621–1626.
[28] Winterbourn, C.C. (1993) Free Radical Biol. Med. 14, 85–90.
[29] Müller, K., Seidel, M., Braun, C., Ziereis, K. and Wiegrebe, W. (1991) Arzneim.-Forsch. 41, 1176–1181.
[30] Kim, K., Rhee, S.G. and Stadtman, E.R. (1985) J. Biol. Chem. 260, 15394–15397.
[31] Stadtman, E.R. and Berlett, B.S. (1991) J. Biol. Chem. 266, 17201–17211.
[32] Hermes-Lima, M., Castilho, R.F., Meinicke, A.R. and Vercesi, A.E. (1995) Mol. Cell. Biochem. 145, 53–60.