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The in vivo and in vitro oxidation of molecular form 1 of biliverdin reductase to molecular form 3 by diamide

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Administration of phenylhydrazine to rats converted molecular form 1 of the liver biliverdin reductase into its disulfide bridged dimer (molecular form 3). This oxidative dimerization was shown not to be mediated by the NAD^+-dependent dehydrogenase [(1984) Biochem. Biophys. Res. Commun. 121, 249–254]. Administration of diamide produced the same conversion. Although hepatic levels of GSH also decreased, no mixed disulfides of the reductase and GSH could be detected. Administration of the antioxidants allopurinol and α-tocopherol together with the diamide did not affect this conversion of molecular forms produced by the latter. The diamide also oxidized molecular form 1 of biliverdin reductase in vitro and molecular form 3 was formed. The chemical oxidation took place at a high rate and was partially inhibited by GSH but not by cysteine.

Biliverdin reductase; Diamide; Protein oxidation; Phenylhydrazine; Thiol regulation; Rat liver

1. INTRODUCTION

Heme is degraded by heme oxygenase, a microsomal enzyme which selectively oxidizes the α-methine bridge of heme to give biliverdin IXα. The latter is reduced in mammals by a cytosolic biliverdin reductase to bilirubin IXα [1]. In rat liver, biliverdin reductase is present in two molecular forms; a major molecular form 1 (MF1) with a high reduction rate for biliverdin IXα, and a minor molecular form 2 with similar reduction rates for biliverdins IXα and IXβ [2]. When hemoprotein degradation is enhanced by administration of $CoCl_2$ or phenylhydrazine, the pattern of the molecular forms of liver biliverdin reductase changes: molecular form 1 disappears and a new molecular form 3 (MF3) appears [3]. The latter is a dimeric form 1 and is formed by the oxidation of a thiol residue of molecular form 1 to form a disulfide bridge in molecular form 3. In $CoCl_2$ treated rats this oxidative dimerization is mediated by a NAD^+-dependent peroxisomal dehydrogenase which oxidizes molecular form 1 of rat liver biliverdin reductase but is inactive toward biliverdin reductase from spleen or kidney [4]. These consist of only one molecular form, which is similar to form 1 of the liver but differ from it in significant structural details [5].

When the mode of action of the phenylhydrazine on the interconversion of the molecular forms was probed, no induction of the dehydrogenase could be detected (see section 3). Therefore the action of phenylhydrazine had to be attributed to its in vivo conversion to a phenyldiazene, which has been proposed as the biologically active form of phenylhydrazine [6]. It was therefore decided to explore the possibility that diamide (1,1′-azobis(*N,N*′-dimethylformamide), could induce the thiol-disulfide interconversion of the molecular forms of biliverdin reductase. Diamide is a stable diazene which does not give rise to free radicals, and is known to penetrate cells rapidly and to oxidize glutathione both in vivo and in vitro [7]. We report below that administration of diamide to rats resulted in the conversion of molecular form 1 of liver biliverdin reductase into molecular form 3, and that it was not found to affect the kidney or spleen enzymes. Diamide was also found to dimerize molecular form 1 to molecular form 3 in vitro. Comparison of molecular form 3 obtained by oxidation with diamide (either in vivo or in vitro) with the same molecular form obtained by the action of the dehydrogenase on molecular form 1, showed that it is the same protein.

2. MATERIALS AND METHODS

2.1. *Materials*

Biliverdin IXα and IXβ were prepared by the chemical oxidation of hemin IX and separated as described elsewhere [8]. NADPH, NAD^+, cysteine, GSH and α-tocopherol were from Sigma Chemical Co. Diamide (1,1′-azobis(*N,N*′-dimethylformamide)) and phenylhydrazine were from Aldrich. All the other chemicals and solvents used were of the highest analytical grade.

2.2. *Animals*

Female albino Wistar rats (180–200 g) were used. They were injected s.c. with a freshly prepared 0.2% phenylhydrazine saline solu-

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tion (0.2 ml/100 g body wt) and then fasted till the time of sacrifice. When rats were treated with diamide, the latter was injected i.p., dissolved in saline solution at the concentration indicated in each case. When α-tocopherol (1.5 mg/100 g) or GSH (1.5 mg/100 g) were administered to the rats together with the diamide, they were injected i.p. 15 min before the latter. Control animals were injected with saline solution and fasted before sacrifice.

2.3. *Enzyme preparations*

Molecular form 1 of biliverdin reductase was obtained from the liver of control rats and purified to homogeneity as described elsewhere [5]. Molecular form 3 of biliverdin reductase was obtained from $CoCl_2$ treated rats and purified as described [9]. The peroxisomal NAD^+-dependent dehydrogenase was prepared as described elsewhere [4]. Purification of the liver peroxisomes was performed according to Singh et al. [10].

2.4. *Enzyme assay*

The converting enzyme was assayed in the peroxisomal liver preparations by using the purified molecular form 1 as substrate and then by measuring reductase activity on biliverdins IXα and IXβ as described elsewhere [3]. The transformation of form 1 into form 3 was measured by preincubating in a final volume of 100 μl: 10 μmol of phosphate buffer (pH 7.4), biliverdin reductase (MF1) (200 ng), NAD^+ (100 μmol) and 25 μl (250 μg) of the peroxisomal suspension. The mixture was incubated 10 min at 37°C, and applied to a DEAE-cellulose column (1 × 15 cm). Both molecular forms were separated using a phosphate buffer (pH 7.4) gradient [3]. In control runs the peroxisomal suspension was omitted and only form 1 was eluted from the DEAE-cellulose; its activity was taken to be 100%. In the presence of the peroxisomes the conversion of form 1 into form 3 was measured by the decrease in the activity of the former and the simultaneous increase in the activity of the latter. When the diamide was used to perform the transformation, the incubation mixture was the same except that the NAD^+ and the peroxisomal fraction were substituted by the diamide.

Biliverdin reductase activity was assayed at 37°C for 10 min. The incubation mixture contained in a final volume of 100 μl: 10 mM potassium phosphate (pH 7.4), 500 μM NADPH, 13 μM of biliverdin IXα or biliverdin IXβ (usually the experiments with both isomers were run in parallel when form 3 was measured), and enzyme (200 ng, spec. act. 3500 units/mg protein), unless otherwise indicated. The activities were determined by measuring the formation of bilirubin which was estimated from the $\Delta A_{455\,nm}$. This difference was found to be proportional to the concentration of bilirubin. An $\epsilon = 50\ mM^{-1} \cdot cm^{-1}$ ($vis_{max} = 455$ nm) was used for bilirubin estimation.

2.5. *Analytical procedures*

Protein concentration was determined by the method of Bradford [11] using bovine serum albumin as standard. Reduced glutathione was measured spectrofluorometrically using o-phthalaldehyde according to the method of Hissin and Hilf [12]. Native polyacrylamide gel electrophoresis was performed on 7.5% polyacrylamide gels using the neutral discontinuous buffer system of Williams and Reisfeld [13]. For protein detection gels were stained with silver nitrate according to the method of Merril et al. [14].

3. RESULTS

3.1. *Formation of molecular form 3 of rat liver biliverdin reductase in phenylhydrazine-treated rats*

Rats injected with phenylhydrazine were sacrificed at different time intervals after the administration of a single dose of the base. The livers were assayed for the appearance of the NAD^+-dependent dehydrogenase and for the activity of molecular forms 1 and 3 of biliverdin reductase. The levels of reduced glutathione (GSH) were also measured on liver samples. As can be seen in Table I, form 3 started to appear 3 h after the phenylhydrazine injection simultaneously with the disappearance of form 1. The conversion was complete after 5 h. The levels of GSH in the livers started to decrease simultaneously with the appearance of form 3. No activity of the NAD^+-dependent dehydrogenase could be detected in the liver at any time interval (up to 18 h) after phenylhydrazine injection.

3.2. *In vivo formation of molecular form 3 of rat liver biliverdin reductase in diamide-treated rats*

Diamide was administered to rats at different doses starting with 0.25 mg/100 g body weight up to 2.5 mg/100 g body weight. The rats were sacrificed 3 h after administration of the chemical. Higher concentrations of diamide led to the transformation of form 1 of rat liver biliverdin reductase into form 3 (Fig. 1). At a diamide concentration of 1.5 mg/100 g of body weight, a 100% conversion of form 1 into form 3 was achieved. To exclude the possibility that mixed disulfides of form 1 and GSH were formed, the liver preparations were filtered through a Sephadex G-100 column equilibrated and eluted with 100 mM phosphate buffer (pH 7.4)–2 M KCl. Molecular form 3 (68 kDa) was the only active biliverdin reductase peak, thus excluding the possibility that a mixed disulfide was formed. The presence of an inactive mixed MF1-SSG disulfide was excluded since no immunoreactive species could be detected using anti-MF1 polyclonal antibodies which do not detect MF3 [15]. The decrease in the levels of liver GSH did not show a strict correlation with the interconversion of the

Table I

In vivo transformation of molecular form 1 of biliverdin reductase into molecular form 3 and decrease in GSH content in the livers of rats treated with phenylhydrazine

Time after treatment (h)	Biliverdin reductase activity (% of control)		GSH content (% of control)
	MF1	MF3	
Control	100	0	100
1	100	0	100
2	100	0	90
3	85	15	63
5	0	100	44
9	0	100	42
18	0	100	42

The 150000 × g supernatant of the liver extract (0.5 ml) was applied on a DEAE-cellulose column (1 × 15 cm) and the molecular forms of biliverdin reductase were separated as described [3]. A 100% of MF1 or MF3 activity was equivalent to 24 ± 1.8 nmol of bilirubin formed/5 min. The GSH content of the control livers was 6.7 ± 0.8 μmol/g liver. The results are the mean values ± SD of three determinations using three rats each time

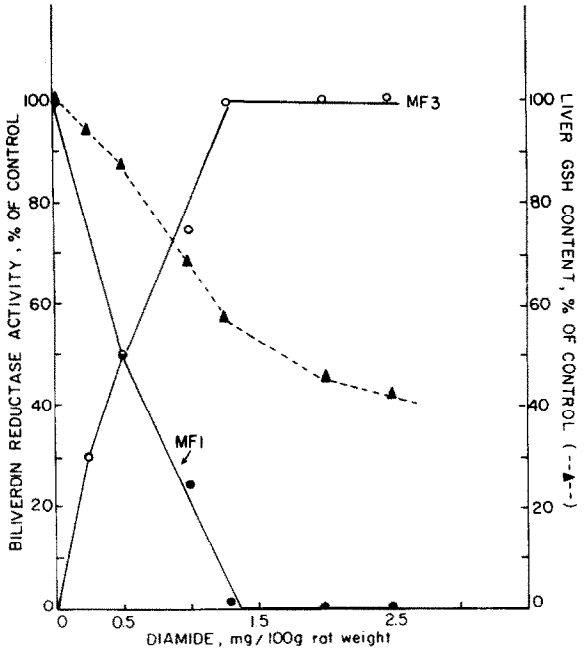

Fig. 1. Effect of increasing diamide doses on the intrahepatic transformation of molecular form 1 of biliverdin reductase into molecular form 3 and on the liver GSH content. Experimental conditions as well as the assay of biliverdin reductase activities and the GSH content are described in Table I.

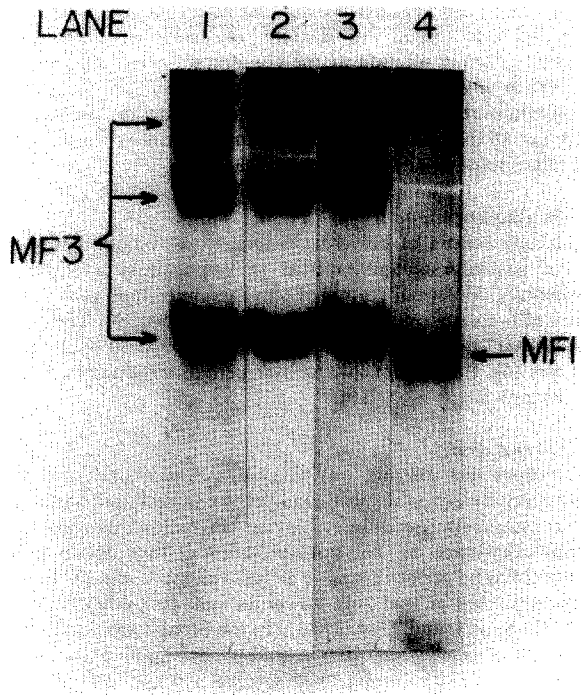

Fig. 2. Electrophoretic analysis by native PAGE of molecular form 3 of biliverdin reductase formed in the livers of rats treated with: lane 1, $CoCl_2$; lane 2, phenylhydrazine, 12 h; lane 3, diamide (1.25 mg/100 g rat body weight). Lane 4 is molecular form 1 of biliverdin reductase. The enzymes were separated and purified as described in section 2; 75 ng of protein were applied and stained with silver nitrate. Form 3 shows two additional bands (associated forms) in addition to the main band.

molecular forms; only higher diamide concentration led to a decrease of GSH levels which reached values of about 50% of the normal ones (Fig. 1). In the livers of rats sacrificed 12 h after the diamide treatment (1.25 and 2.5 mg/100 g body weight) the levels of GSH and the content of form 3 were the same as those found 3 h after the injection of the chemical.

When the antioxidants allopurinol and α-tocopherol (1.5 mg/100 g body weight) were administered together with the diamide (1.25 mg/100 g body weight) and the livers were excised 12 h later, essentially the same results as those obtained in the absence of the antioxidant were found. Hence, active oxygen species or radicals are apparently not involved in the diamide induced conversion of form 1 into form 3. The diamide was not found to induce the appearance of the NAD^+-dependent peroxisomal dehydrogenase.

Administration of the diamide (1.25 mg/100 g body weight) together with GSH (1.5 mg/100 g body weight), prevented the transformation of form 1 into form 3. Intrahepatic GSH levels decreased only 15% as compared to the control values indicating that the diamide did not reach the liver, very likely due to its reaction with the circulating GSH.

Biliverdin reductase of kidney and spleen was not affected by the diamide treatment and therefore behaved toward this chemical in a similar manner as toward the peroxisomal dehydrogenase.

The form 3 produced in vivo by diamide was found to be identical with that formed by the phenylhydrazine treatment and by the $CoCl_2$ induced peroxisomal dehydrogenase. The form 3 of the three sources was purified and compared by PAGE electrophoresis (Fig. 2, lanes 1–3).

3.3. *Chemical oxidation of molecular form 1 of biliverdin reductase to molecular form 3 by diamide*

In order to establish if the intrahepatic dimerization of form 1 to form 3 caused by the diamide is a chemical oxidation, the reaction was carried out directly in vitro. Purified form 1 of rat liver reductase as well as biliverdin reductase from kidney and spleen, were incubated with increasing concentrations of the diamide (Fig. 3A). Formation of form 3 (at the expense of form 1) increased linearly with diamide concentration between 0.1 mM and 0.4 mM for a 5 μM enzyme concentration. The rate of form 3 formation was fast at a 0.35 mM diamide concentration and reached 100% in 10 min (Fig. 3B). The transformation of form 1 into form 3 could be followed by native PAGE (Fig. 3B, inset). GSH competed with the oxidation of form 1 by diamide. When both were added together to a 0.35 mM diamide concentration, a 50% inhibition in the formation of form 3 was achieved at a 30 μM GSH concentration (Fig. 3C). When the latter was added 2 min after

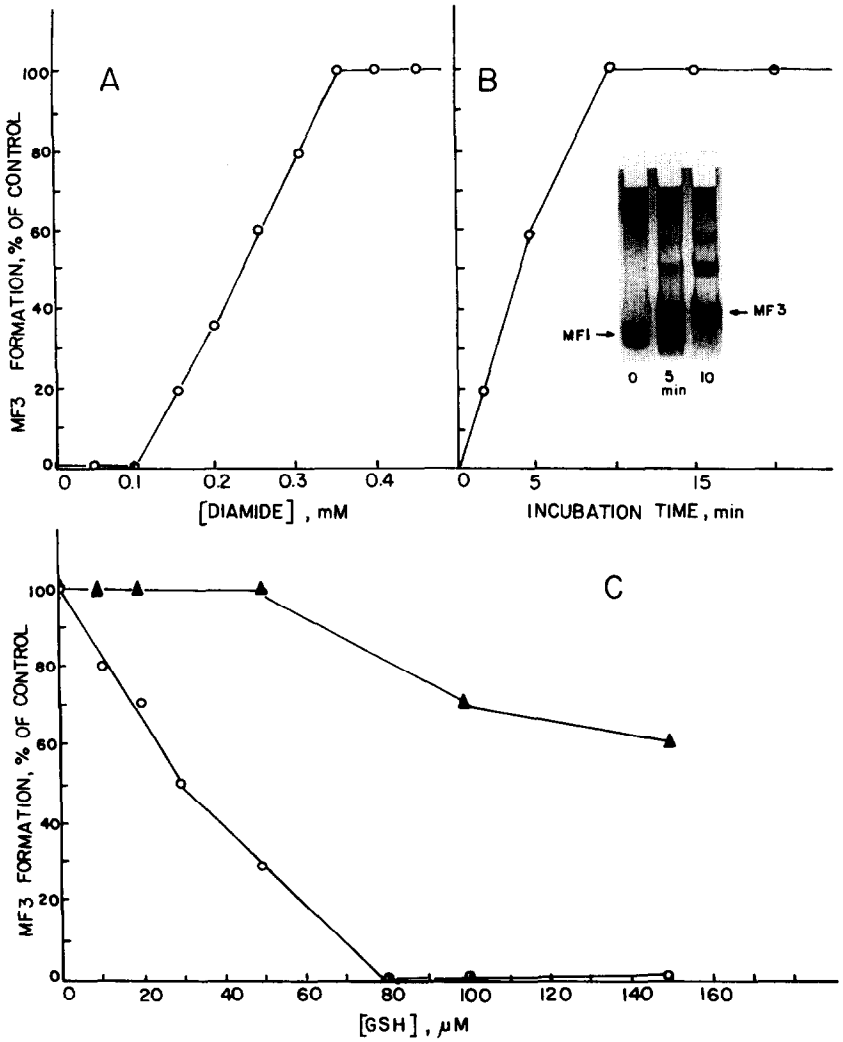

Fig. 3. In vitro transformation of molecular form 1 of biliverdin reductase into molecular form 3 by diamide. (A) Concentration-dependent transformation of form 1 into form 3. The enzyme (form 1, 36 μM) was incubated for 10 min at 37°C with the indicated diamide concentrations and the incubation mixture was then analysed for the biliverdin reductase molecular forms. (B) Rate of MF3 formation in the presence of 0.35 mM diamide. Inset: transformation of form 1 into form 3 as a function of time as analysed by native PAGE. (C) Inhibition of the diamide (0.35 mM)-induced oxidation of form 1 (3.6 μM) by increasing concentration of GSH (○) and cysteine (△).

mixing the enzyme with the diamide, a 50% inhibition was reached at a 50 μM GSH concentration (data not shown). Cysteine competed only to a small extent with form 1 for the diamide (Fig. 3C), a fact which agrees with the low rate constant found for the reaction of diamide with cysteine [16]. The biliverdin reductases of spleen and kidney were not oxidized by diamide.

4. DISCUSSION

The primary target for diamide within the cells is GSH which is oxidized to GSSG [6]. Very few thiol proteins were also found to be directly oxidized by the diamide [17–19]. The in vivo oxidation of form 1 of biliverdin reductase by diamide is therefore a unique example of a thiol protein which is rapidly oxidized even in the presence of GSH (Figs 1 and 3C). Although the diamide was found to oxidize intrahepatic GSH (Table I), thiol-disulfide conversion for biliverdin reductase was fast and specific enough as to avoid formation of mixed disulfides with GSH [17]. The high reactivity toward nucleophilic reagents of this thiol group of form 1 has already been noted [15].

The biological implications of the oxidations of form 1 to form 3 are many. In the disulfide form (MF3), the thiol of the active side is less accessible to thiol reagents and is more protected from the attack by oxidizing species. The kinetics and specificity of the enzyme also changes; while form 1 reduces biliverdin IXα at a higher rate than biliverdin IXβ, form 3 reduces both isomers at similar high rates [3]. It has been established that under conditions of oxidative stress, heme oxygenase is induced and hemoproteins are degraded. Phenylhydrazine in the presence of oxygen is known to

degrade hemoproteins by chemical attack, and it is also known that the chemical oxidation of hemoglobin results mainly in the formation of biliverdins IXα and IXβ [20]. The increased excretion of bilirubin IXβ found under conditions of oxidative stress [21] falls in line with the need for an increased reduction rate of biliverdin IXβ. The results reported in this paper suggest that an in vivo formed diazene will lead to the formation in the liver of a form of biliverdin reductase which will be less susceptible to oxidative attack, while at the same time it will reduce more efficiently the β-isomer of biliverdin. It is also an example of the direct oxidation of a protein thiol by diamide in vivo, independent of the action of the latter on the intracellular GSH pool.

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