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Biochimica et Biophysica Acta 1366 (1998) 265–274

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The role of mitochondrial glutathione in DNA base oxidation

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Received 13 May 1998; accepted 21 May 1998

Abstract

The objective of this study was to elucidate the role of mitochondrial GSH in the reactions leading to mitochondrial DNA oxidative damage in terms of 8-hydroxy-desoxyguanosine (8-HOdG) accumulation. With this purpose, tightly coupled mitochondria depleted of matrix GSH were used and the effects of H_2O_2 (generated during the oxidation of substrates) on 8-HOdG levels were investigated. Mitochondrial integrity, assessed by O_2 uptake, respiratory control and P/O ratios, was conserved upon depletion of GSH up to 95%. The rates of H_2O_2 production linked to the oxidation of endogenous substrates by control and GSH-depleted mitochondria were similar. Succinate (in the absence or presence of antimycin A) enhanced the rate H_2O_2 production to a similar extent in both control and GSH-depleted mitochondria. These rates of H_2O_2 production accounted for 1.5–2.5% of the rate of O_2 uptake. The levels of 8-HOdG in GSH-depleted mitochondria were 35–50% lower than those in control mitochondria, when measured at different H_2O_2 production rates. Conversely, in experiments carried out with calf thymus DNA with different Cu/Fe content, GSH increased 1.4–2.4-fold the accumulation of 8-HOdG. These values were further enhanced (44–50%) by superoxide dismutase and decreased by catalase. The lower levels of 8-HOdG in GSH-depleted mitochondria and the higher levels in GSH-supplemented calf thymus DNA suggest a role for the non-protein thiol in the reactions leading to mtDNA oxidative damage. These findings are interpreted in terms of the redox transitions involving O_2, GSH, and metal catalysts bound to DNA. A mechanism is proposed by which GSH plays a critical role in the reduction of DNA-Cu complexes and decays by free radical pathways kinetically regulated by superoxide dismutase.

Keywords: Mitochondrial DNA; 8-Hydroxy-desoxyguanosine; Glutathione; Hydrogen peroxide; Oxidative stress; Mitochondria

1. Introduction

Mitochondria are major cellular sources of H_2O_2 originating from the disproportionation of $O_2^{\bullet-}$ produced during electron flow through the respiratory chain. Approximately, 1–3% of the total O_2 consumed by mitochondria may be accounted for in terms of H_2O_2 production [1,2]. These oxidants appear to elicit a sustained damage to mitochondria leading to deficits in mitochondrial function [3]. Accordingly, correlations have been established between the H_2O_2 formed during the oxidation of substrates by mitochondria, an enhanced protein carbonyl content [4,5], and mitochondrial DNA (mtDNA) base oxidation and strand breaks [6,7].

Mitochondrial DNA is highly susceptible to oxidative damage [8–10] and the amount of 8-hydroxy-

Abbreviations: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; GSH, glutathione; GSSG, glutathione disulfide; 8-HOdG, 8-hydroxy-desoxyguanosine; dG, desoxyguanosine; DMSO, dimethylsulfoxide

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0005-2728/98/$ – see front matter
PII: S0005-2728(98)00125-X

desoxyguanosine (8-HOdG), a biomarker of DNA oxidative lesions [8,11], is 10–20-fold higher in mtDNA than in nuclear DNA [12]. Damage to mtDNA gains further significance in view of the fact that mtDNA encodes several proteins essential for the respiratory chain function. Thus, mtDNA mutations may be expected to lead to loss of mitochondrial integrity [9].

The formation of 8-HOdG in mtDNA also raises important questions concerning its repair [13], association with mitochondrial dysfunctions, and prevention by antioxidants [14]. It is reasonable to assume that mtDNA base oxidation reflects an alteration of the equilibrium established by the continuous production of H_2O_2 by the respiratory chain and its removal by mitochondrial glutathione peroxidase [15]. GSH, present in the mitochondrial matrix in high concentrations (6–10 mM) [16], serves as cosubstrate for the latter reaction. Hence, it may be expected that loss of mitochondrial GSH would be harmful for mitochondrial functions particularly under conditions of oxidative stress [16]. Furthermore, the non-enzymic reactions of this abundant thiol with oxygen-, carbon-, and nitrogen-centered radicals [17] broaden the antioxidant range of this molecule. Paradoxically, the antioxidant-derived radical (thiyl radical) originating from these interactions is a powerful oxidant ($E^{o\prime} \sim 0.9$ V [18]), which is capable of initiating peroxidation of lipids [19]. The reducing versatility of GSH in combination with its high concentration in the mitochondrial matrix may actually contribute to oxidative stress. This notion is analogous to a reductive stress [20–22] entailing the mobilization of iron from intracellular stores and its further participation in free radical reactions leading to oxidative damage of biomolecules.

The dichotomy of GSH actions described above compelled us to undertake this study, which was aimed at elucidating the role of mitochondrial matrix GSH in the reactions leading to mtDNA base oxidation assessed in terms of 8-HOdG. With this purpose, changes in mtDNA 8-HOdG levels were assessed under conditions of H_2O_2 generation during the oxidation of substrates by tightly coupled mitochondria depleted of GSH. In an alternative experimental approach, the effect(s) of GSH on 8-HOdG levels were examined in calf thymus DNA supplemented with GSH under conditions involving changes in metal catalysis and $O_2^{\bullet-}$-dependent free radical reactions.

2. Materials and methods

2.1. Chemicals and biochemicals

Proteinase K, RNase T1 from *Aspergillus oryzae*, Nuclease P1 from *Penicillium citrium*, calf thymus DNA, fatty-acid free bovine serum albumin, nucleosides, ADP, antimycin, cysteine, cystine, and chlorodinitrobenzene were from Sigma (St. Louis, MO). GSH and GSSG were obtained from Fluka (Buchs, Switzerland). Bovine pancreas RNase A, calf intestine alkaline phosphatase, and horseradish peroxidase were from Boehringer (Mannheim, Germany). 8-HOdG was synthesized according to [23]. All other reagents were of analytical grade.

2.2. Isolation of mitochondria and depletion of mitochondrial GSH

Rat liver mitochondria from Wistar male rats (180–220 g body weight) were isolated as previously described [24] using 0.21 M mannitol/0.07 M sucrose/0.1 mM sodium EDTA/10 mM Tris-phosphate, pH 7.6 (MSTE) as isolation buffer. For depletion of mitochondrial GSH, isolated mitochondria (10–12 mg/ml) suspended in MSTE were incubated with 100 μM 1-chloro-2,4-dinitrobenzene in ethanol for 2 min at room temperature [25]. Following centrifugation, the pellets were washed twice with MSTE to remove any excess of reagent. Control mitochondria were incubated with an equimolar amount of ethanol.

2.3. Determination of Cu content in calf thymus DNA

Calf thymus DNA, dissolved in PBS buffer at a concentration of 1 mg/ml was dialyzed overnight against PBS (control) or PBS plus 1 mM bathocuproine disulfonate (bathocuproine) at 4°C using a dialysis membrane of 8000–12 000 MW cut-off. Cu content (determined by furnace graphite atomic absorption) of control and dialyzed calf thymus DNA was 0.05 and 0.023 nmol/mg DNA, respectively. Fe levels, also affected by this treatment, were 0.091 and 0.027 nmol/mg DNA in control and dialyzed DNA,

Table 1
Sulfur-containing compounds in control and GSH-depleted rat liver mitochondria

Sulfur-containing compounds	Control (nmol/mg protein)	GSH-depleted (nmol/mg protein)
GSH	6.00 ± 1.00	0.27 ± 0.08
GSSG	0.30 ± 0.02	0.13 ± 0.05
Cysteine	0.83 ± 0.05	0.48 ± 0.03
Cystine	0.08 ± 0.01	0.31 ± 0.05
Mixed disulfide Cys-SG	0.22 ± 0.05	0.11 ± 0.03

respectively. In a typical experiment, calf thymus DNA (1 mg/ml) was supplemented with the indicated reagents for the selected time and incubations were terminated by the addition of 0.3 M sodium acetate and mixed with 2 vols. absolute ethanol (cooled at −20°C).

2.4. Treatment of DNA samples for 8-HOdG measurements

Mitochondria were subjected to two procedures for 8-HOdG extraction from mtDNA: phenol/chloroform treatment [11] and pronase/ethanol precipitation [26].

2.5. Phenol/chloroform extraction

Control and treated mitochondria were centrifuged at 9000 rpm for 10 min to remove the supernatant. The pellet was resuspended to a protein concentration of 10 mg/ml with 1.7 ml 10 mM Tris-HCl/100 mM EDTA, pH 8.0. This suspension was supplemented with 860 μl 10% SDS plus 34 μl of 5 mg/ml proteinase K and incubated at 37°C for 2 h. The solution was extracted twice (v/v) with saturated phenol (0.1% w/w 8-hydroquinoline, 0.5 M Tris-HCl, pH 8.0) and also twice with chloroform (v/v). (Phenol was freshly distilled and kept cold, in the dark, and in a N_2 atmosphere until use; 8-hydroxyquinoline was recrystallized twice before use.) DNA was precipitated in 2 vols. of ethanol with 0.3 M sodium acetate for 20 h at −20°C. The pellet was washed with 170 μl of 75% ethanol, centrifuged, and resuspended in 200 μl of TE buffer (10 mM Tris-HCl, pH 7.5/EDTA 1 mM). To this solution, 4 μl of RNAse A/T1 mix (50 U/ml RNase A and RNase T1 100 U/ml in TE buffer) was added and incubated for 30 min at 37°C. This solution was extracted once with phenol and twice with chloroform. DNA was precipi-

Table 2
Functional parameters of, and hydrogen peroxide production by, control and GSH-depleted mitochondria

Parameters	Control	GSH-depleted
Oxygen consumption (O atom/min/mg protein)		
State 4	13.8 ± 1.0	16.1 ± 0.7
State 3	44.0 ± 2.0	48.0 ± 4.0
Respiratory control ratio	3.6 ± 0.2	3.4 ± 0.3
P/O ratio (Pi consumed per O atom reduced)	1.7 ± 0.3	1.6 ± 0.2
H_2O_2 production (nmol/min/mg protein)		
Endogenous substrate	0.05 ± 0.03	0.04 ± 0.01
Plus succinate	0.33 ± 0.08	0.24 ± 0.04
Plus succinate+antimycin	0.70 ± 0.10	0.70 ± 0.20
$[H_2O_2]_{ss}$ (matrix) (10^{-8} M)		
Endogenous substrate	1.5	2.2
Plus succinate	6.9	6.8

O_2 consumption in state 4 was measured in reaction mixtures consisting of 1 mg mitochondrial protein/ml in MSTE buffer (as described in the Section 2) and 10 mM succinate. State 3 was measured in the same conditions in the presence of 0.1 mM ADP. The reaction medium utilized for H_2O_2 production contained 1 mg mitochondrial protein/ml in MSTE supplemented with either 2 mM $MgCl_2$ (endogenous substrate), or 10 mM succinate, or 10 mM succinate plus 50 nM antimycin A (in DMSO). Other assay conditions as described in Section 2.

Table 3
Comparison of two DNA isolation procedures in terms 8-hydroxy-desoxyguanosine levels and DNA yield

	Method of DNA extraction			
	Phenol/chloroform		Pronase E/ethanol	
	Control	GSH-depleted	Control	GSH-depleted
8-HOdG (per 10^6 dG)	61 ± 3	48 ± 5	6.3 ± 0.3	4.1 ± 0.07
(fmol/μg mtDNA)	37 ± 2	29 ± 3	3.8 ± 0.2	2.4 ± 0.04
DNA yield (μg mtDNA/liver)	68 ± 20	61 ± 10	72 ± 5.0	70 ± 17

Experimental conditions as described in Section 2.

tated in 2.5 vols. of ethanol with 0.3 M sodium acetate (pH 5.0) and kept on dry ice for 1 h. The pellet was recovered by centrifugation and washed with 75% ethanol at −20°C. The pellet, resuspended in 200 μl of 20 mM sodium acetate, pH 4.8, was hydrolyzed enzymatically as described below.

2.6. Pronase E/ethanol extraction

Several reports showed that the phenol-chloroform extraction yielded a DNA preparation more prone to oxidation (the frequency of 8-HOdG increases in 2–60-fold [27,28], especially following UV irradiation [29,30]). Therefore, we used an alternative procedure which recovers DNA in comparable yields to the phenol-chloroform extraction and is likely not to introduce artifactual oxidative damage.

This technique was adapted from Kendall et al. [26] including the modifications suggested by Adachi et al. [31]. Control and treated mitochondria were resuspended in 3.5 ml of buffer 1 (20 mM NaCl, 5 mM trisodium citrate, pH 6.5) under vigorous vortexing. To this solution, 0.5 ml of 20 mg/ml RNase- and DNase-free pronase E in buffer 1 plus 4 ml of buffer 2 (20 mM Tris; 20 mM EDTA; 1.5% sarkosyl, pH 8.5) were added, and where indicated, 0.5 ml of 5% butylhydroxytoluene in methanol was added to this solution. After 6 h at 45°C in a N_2 atmosphere, 4 ml of buffer 3 (10 mM Tris-HCl; 1 mM EDTA, pH 7.5) and 1 ml of 7.5 M ammonium acetate were added and mixed by inversion. The DNA was precipitated by adding 70% ethanol at −20°C (approximately 30 ml), and then stored on dry ice for 30 min. The precipitated DNA (recovered by centrifugation) was rinsed with 70% ethanol. This DNA pellet was dried under N_2, then resuspended in 200 μl of 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, and treated with DNase-free RNase. The RNA-free DNA was recovered by centrifugation in Centricom 10k, and washed subsequently with 65, 75, and 95% ethanol.

8-HOdG extraction from calf thymus DNA (type I) was carried out by the pronase/ethanol method. The mtDNA pellet obtained from either extraction procedure was hydrolyzed enzymically as described [32,33]. Occasionally, the resulting DNA from either procedure was stored overnight at −80°C in 95% ethanol containing 0.01% BHT (without noticeable increase in 8-HOdG) and then subjected to hydrolysis.

Table 4
Levels of 8-hydroxy-desoxyguanosine in DNA from mitochondria incubated under different experimental conditions

Conditions	[8-Hydroxy-desoxyguanosine] (fmol/μg DNA)	
	Control	GSH-depleted
No substrate	3.80 ± 0.2	2.44 ± 0.04
Plus succinate	5.56 ± 0.3	3.07 ± 0.02
Plus succinate/antimycin A	6.06 ± 0.3	3.62 ± 0.06

Rat liver mitochondria (control and GSH-depleted) were incubated with no substrate, plus succinate, and plus succinate in the presence of antimycin (as described in the legend to Table 2). After 30 min at room temperature, the mtDNA was isolated following the pronase E/ethanol precipitation procedure and hydrolyzed by means of nuclease P1 and alkaline phosphatase. Nucleosides were separated by HPLC with UV and electrochemical detection as described in Section 2. The amount of DNA before hydrolysis was measured by fluorescence as described in Section 2, while after hydrolysis was calculated from the amount of dG/μg rat liver mtDNA.

Table 5
Levels of 8-hydroxy-desoxyguanosine in calf thymus DNA with different copper content

Additions	[8-Hydroxy-desoxyguanosine] (fmol/μg DNA)	
	Control	Bathocuproine
None	8.2 ± 0.8	5.7 ± 0.6
GSH	15.0 ± 1.0	13.6 ± 1.4
GSH+superoxide dismutase	19.5 ± 2.2	17.5 ± 1.6
GSH+catalase	14.1 ± 1.1	7.7 ± 0.8

The reaction mixture consisted of 1 mg/ml calf thymus DNA (control or dialyzed against bathocuproine) and 25 μM H_2O_2 in PBS in the absence or presence of 1 mM GSH, 1 mM GSH plus 5 μM superoxide dismutase, or 1 mM GSH plus 50 μM catalase. These mixtures were incubated for 30 min at room temperature, DNA was extracted using the pronase E/ethanol procedure and hydrolyzed as described in Section 2. Copper content of control and dialyzed calf thymus DNA was 0.05 and 0.023 nmol/mg DNA, respectively.

DNA digestion to nucleoside level was carried out using the method of Kasai et al. [32] considering the modifications introduced by Floyd et al. [33]. The DNA (isolated by either method) was recovered by centrifugation and dried under N_2. The pellets were resuspended in SSC (5 mM sodium citrate, 20 mM NaCl, pH 6.5) and diluted up to 2 μg/μl with SSC. DNA concentration was measured by absorbance at 260 nm or using Hoecht dye on a TKO100 fluorometer (λ_{exc} = 365 nm, λ_{em} = 460 nm) according to the manufacturer's directions (Hoefer Scientific Instruments, San Francisco, CA)). The samples were heat-denatured (95°C for 10 min), and then were hydrolyzed to nucleosides by adding 2 μl of 3 M sodium acetate, pH 4.8, 100 μM $ZnCl_2$ and 24 μl of Nuclease P1 (1 mg/ml in 20 mM sodium acetate, pH 4.8). After 15 min at 65°C, 12 μl 2 M Tris-HCl, pH 8.0, was added, followed by 4 μl alkaline phosphatase. The incubation was performed at 37°C for 1 h, and then 30 μl of 3 M sodium acetate, pH 4.8 plus 1 mM desferal were added. The samples were chromatographed as soon as possible because an increase in 8-HOdG at a rate of 0.14 ± 0.03 h^{-1} $(10^5$ dG$)^{-1}$ was observed if the nucleosides were stored at 0–4°C. The resulting nucleosides were separated by HPLC (see below).

2.7. High performance liquid chromatography (HPLC) assays

Nucleosides were separated by HPLC [34] and measured using a diode array detector (set at 260 and 290 nm) in series with an electrochemical detector (set at +450 mV). The amount of 8-HOdG was quantified using a synthetic 8-HOdG standard (measured by electrochemical detection) and referred to the total amount of dG (measured by UV detection). Sulfur-containing compounds were identified in reactions mixtures consisting of mitochondria (2–3 mg/ml from control and GSH-depleted samples) supplemented with 1 mM γ-Glu-Glu, 1 mM bathophenanthroline disulfonate, and 10% of perchloric acid and frozen in liquid nitrogen. Samples, maintained overnight at −70°C, were centrifuged at 5000 rpm for 15 min and aliquots (0.5 ml) of the supernatant were derivatized essentially as described [35]. Identification of thioether derivatives was performed by comparison and co-injection with stand-

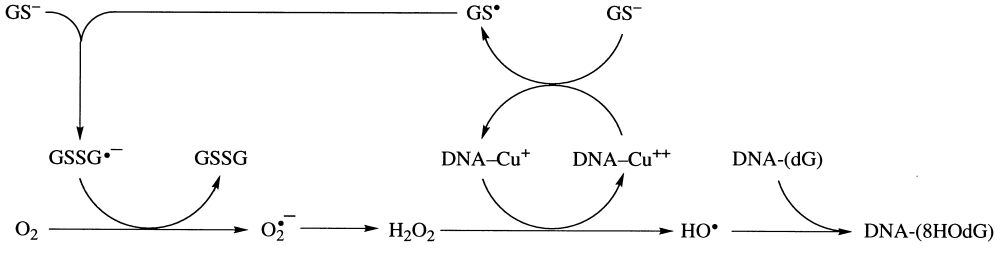

Fig. 1. Proposed mechanism for the enhancing effect of GSH on DNA base oxidation.

ards (GSH, GSSG, Cys, and CySS) derivatized as above [35].

2.8. O_2 consumption and H_2O_2 measurements

O_2 consumption was measured polarographically using a Clark oxygen electrode. P/O ratios were calculated from the total amount of O_2 consumed during state 3 respiration after the addition of a known amount of ADP. H_2O_2 was measured fluorometrically as the horseradish peroxidase-mediated oxidation of scopoletin [36] in mitochondrial suspensions supplemented with 2 mM $MgCl_2$ (control conditions), 10 mM succinate, or succinate plus antimycin (50 nM in DMSO).

2.9. Statistical analysis

Data in Tables 1–5 are expressed as mean ± S.E. and were evaluated by one-way analysis of variance (ANOVA) ($P < 0.1$). Experiments were run in triplicate or quadruplicate with both control and GSH-depleted mitochondria.

3. Results

3.1. Sulfur-containing compounds in control and GSH-depleted mitochondria

Treatment of tightly coupled rat liver mitochondria with chlorodinitrobenzene resulted in ~95% depletion of mitochondrial matrix GSH (Table 1). The effect of this treatment on other mitochondrial sulfur-containing compounds is listed in Table 1: GSSG, cysteine, and mixed disulfides (cys-SG), present in control mitochondria in an amount 3.7–13.8% of that of GSH, were decreased upon treatment with chlorodinitrobenzene by 42–56%. Conversely, cystine was increased ~3.8-fold by the aforementioned treatment. Changes in the mitochondrial sulfur-containing compounds pattern did not affect the parameters of mitochondrial integrity assessed in terms of O_2 consumption in states 4 and 3 and the respiratory control and P/O ratios (Table 2).

The rates of H_2O_2 production originating from the oxidation of either endogenous substrates or succinate (plus or minus antimycin A) by control and GSH-depleted rat liver mitochondria were essentially similar. H_2O_2 formation during the oxidation of succinate accounted for 2.4 and 1.5% of the rate of O_2 uptake in state 4 by control and GSH-depleted mitochondria, respectively (Table 2). The highest rate of H_2O_2 generation obtained during succinate oxidation in the presence of antimycin was not affected by 95% depletion of mitochondrial GSH (Table 2).

The steady-state matrix levels of H_2O_2 ($[H_2O_2]_{ss}$) measured during the oxidation of endogenous substrates were 1.5 and 2.3 nM for control and GSH-depleted mitochondria, respectively. Those measured during the oxidation of succinate were 6.9 and 6.8 nM, respectively (Table 2). The values originating from the oxidation of succinate in the presence of antimycin could not be considered strictly as steady-state concentrations, because the requisite conditions for this type of measurement, i.e. a plateau entailing equal rates of H_2O_2 production and removal, could not be obtained.

In addition to these measured values (Table 2), $[H_2O_2]_{ss}$ was calculated by the steady-state approximation method (in which the rate of production of H_2O_2 equals that of removal under steady-state conditions ($+d[H_2O_2]/dt = -d[H_2O_2]/dt$)) [15,37]. This calculation considers that removal of H_2O_2 in the mitochondrial matrix occurs via glutathione peroxidase (concentration in mitochondria = 1.17×10^{-6} M) catalyzing H_2O_2 reduction at 5×10^{7} M^{-1} s^{-1} [15]. The $[H_2O_2]_{ss}$, thus calculated, was 9.4×10^{-8} M for control mitochondria oxidizing succinate and 6.8×10^{-8} M for GSH-depleted mitochondria. These values are in good agreement with those measured and listed in Table 2.

$$[H_2O_2]_{ss} = \frac{d[H_2O_2]/dt}{k[GPx]} =$$

$$\frac{5.5\times10^{-6}\ M^{-1}\ s^{-1}}{5\times10^{7}\ M^{-1}\ s^{-1}[1.17\times10^{-6}\ M]} = 9.4\times10^{-8}\ M$$

The function of glutathione peroxidase, which overrides any competing H_2O_2 scavenger in the mitochondrial matrix, in GSH-depleted mitochondria merits further comments. Glutathione peroxidase activity is not expected to be affected by this GSH depletion; the remaining GSH (0.27 ± 0.08 mM; Table 1) supports an effective peroxidase activity (its affinity for GSH is in the 0.01–0.02 mM range). Ac-

cordingly, the rate of removal of H_2O_2 from the medium by control and GSH-depleted mitochondria was 26 ± 0.5 and 20 ± 0.3 nmol/min/mg protein, respectively. Hence, the calculation of $[H_2O_2]_{ss}$ in control and GSH-depleted mitochondria described above did not take into account changes in glutathione peroxidase activity. Taken together, these data suggest that there is no impaired removal of H_2O_2 imposed by this GSH depletion.

3.2. Mitochondrial GSH and the levels of mtDNA base oxidation

The level of 8-HOdG was measured in mtDNA and it was expressed as values relative to the amount of dG or as fmol per μg DNA. In the latter instances, a value of 0.6 nmol dG per μg DNA was assumed (DNA composed of 20% dG) [38]. This value is lower than that (0.648) applicable to nuclear DNA [11]. The value for [A+T]/[C+G] obtained with the pronase E/ethanol method was 1.41 ± 0.05, close to that (1.5) previously reported for mtDNA [38].

The levels of 8-HOdG were obtained from mtDNA after following two different extraction procedures based on the use of organic solvents (phenol/chloroform) or pronase E/ethanol precipitation. In both instances, the DNA extracted was hydrolyzed by means of nuclease P1 and alkaline phosphatase. Table 3 summarizes the differences between these extraction methods: whereas the amount of mtDNA afforded by either method was essentially similar, the level of 8-HOdG in mtDNA extracted with phenol/chloroform was found to be 10-fold higher than that in mtDNA extracted with pronase E/ethanol precipitation. This is in agreement with the finding that DNA is more prone to oxidation if extracted with phenol [28], especially after air and/or light exposure [27,29,30] resulting in a DNA preparation with a lower yield of enzymatic digestion [39]. Considering these problems, several modifications were introduced in the procedure to minimize artifactual oxidations: (1) the phenol was freshly distilled and kept cold, dark, and in a nitrogen atmosphere until use; (2) the 8-hydroxyquinoline was recrystallized twice before use; (3) the manipulations with DNA were performed with a minimum exposure to light, and when possible at 0–4°C and under a nitrogen atmosphere; and (4) and all the reagents were purchased with the minimum commercially available content of Fe and Cu. These modifications resulted in values of 8-HOdG lower than those obtained by our (2-times lower; [6]) and other (8-times lower; [8]) laboratories using the phenol/chloroform procedure. The lower values of 8-HOdG obtained in this study truly reflect the effect of an optimized procedure and are not related to a higher contamination of nDNA (that may also had lead to lower values) because these mitochondrial preparations and those obtained before [6] showed similar levels of nDNA contamination as judged by gel electrophoresis. Even in the presence of this contamination, if the concentration of nDNA had been equal to 10× the concentration of mtDNA, the level of 8-HOdG would have been only 1.5–2-times higher because the level of the latter species, and those of other xenobiotic-mediated adducts, in mtDNA are 10–20-times higher that those in nDNA. Further experimental evidence was provided by the changes in levels of 8-HOdG in mtDNA with changes in mtGSH (see below).

Evidently, the pronase/ethanol method allowed the obtention of a mtDNA with lower values of 8-HOdG (Table 3) than those obtained with the phenol-chloroform extraction, even under extremely controlled conditions. The 8-HOdG content in mtDNA obtained by the pronase/ethanol method was about 10-times higher than that obtained with mtDNA from HeLa cells ($[8\text{-HOdG}]/[dG] \times 10^6 = 0.3$) the latter mtDNA was obtained through CsCl density gradient centrifugation without the addition of ethidium bromide [27]. This observation may indicate that either the latter method is superior than the pronase/ethanol procedure to isolate mtDNA and quantify oxidative damage, or that the content of 8-HOdG in rat liver mtDNA is higher than that present in HeLa's mtDNA due to different inherent characteristics of each biological system (e.g. rate of H_2O_2 production, content of metals bound to mtDNA, effectiveness of a repair system for 8-HOdG). The important conclusion is that possibly the damage to mtDNA previously reported was artificially increased during its isolation, especially using phenol-chloroform extractions, or that a particular subset of mtDNA is preferentially extracted during this procedure.

Subsequent measurements of 8-HOdG levels in mtDNA were performed by extraction involving pro-

nase E/ethanol precipitation. Table 3 shows that the levels of 8-HOdG were 1.6-fold higher in mtDNA from control than in that from GSH-depleted mitochondria. This was further examined in both types of mitochondria under conditions involving different rates of H_2O_2 production originating from the metabolism of endogenous substrates, oxidation of succinate, and oxidation of succinate in the presence of antimycin A (Table 4). Under all the conditions listed in Table 4, the accumulation of 8-HOdG was lower (35–50%) in GSH-depleted mitochondria than in control mitochondria. These data suggest a role for GSH in the free radical chain process leading to mtDNA base oxidation in the form of 8-HOdG.

The actual concentration of 8-HOdG in control and GSH-depleted mitochondria oxidizing endogenous substrates may be calculated as 1.57 and 1.01×10^{-9} M, respectively, assuming a concentration of mitochondrial protein of 34 mg/g liver [40] and a concentration of mtDNA of 14 μg/g liver [6].

3.3. Calf thymus DNA base oxidation and GSH

The role of GSH in DNA base oxidation was further assessed with calf thymus DNA under experimental conditions designed to study the effect(s) of metals (bound to DNA) and of antioxidant enzymes (superoxide dismutase and catalase). This model was designed in view of the natural occurrence of copper in cell nuclei [41] and the importance of DNA-bound Cu in the site-specific cleavage of H_2O_2 to HO. and the ensuing DNA base oxidation (Eq. 1):

$$\mathrm{DNA-Cu^{+}+H_2O_2\rightarrow DNA-Cu^{2+}+HO^{-}+HO^{\bullet}} \quad (1)$$

The Cu content, measured by atomic absorption spectroscopy, in control calf thymus DNA was 0.05 nmol/mg DNA, equivalent to 1 Cu per 8–10 dG. Extensive dialysis against bathocuproine decreased the levels of Cu to 0.023 nmol/mg DNA. The purpose of dialysis is not necessarily to prevent Eq. 1, but to ensure its regiospecificity by removing metals in solution or loosely bound. It is expected that the metals not removed by dialysis have a high affinity for particular sites in DNA, which may be critical for further nucleobase oxidation.

Calf thymus DNA dialyzed against bathocuproine exhibited a lower baseline level of 8-HOdG (5.7 fmol/μg DNA against 8.2 fmol/μg DNA in untreated calf thymus DNA). Dialysis with bathocuproine removes approximately half the copper in calf thymus DNA, but not that is apparently critical to cause DNA base oxidation: a 54% decrease in copper content was associated with a 30% decrease in 8-HOdG levels (Table 5).

Notwithstanding the relative metal content, GSH increased by 1.4–2.4-fold the level of 8-HOdG in calf thymus DNA. These levels were further enhanced (30–50%) by superoxide dismutase and decreased (44–50%) by catalase. The effect of the former enzyme may be accounted for by a displacement of the equilibrium of reactions yielding $O_2^{\bullet-}$ (see Section 4), whereas the lower levels of 8-HOdG in the presence of catalase may be explained under the terms of Eq. 1 above.

4. Discussion

We have previously shown that the extent of mtDNA nucleobase oxidation (as 8-HOdG) and mtDNA strand breaks was increased by the production of H_2O_2 originating from inner (respiratory chain) or outer (monoamine oxidase catalysis) membrane activities [6,7]. Although mitochondrial membranes do generate $HO^{\bullet}$ [6], it is unlikely that this species formed in the bulk solution, given its high chemical reactivity and low diffusion ratio, is directly involved in mtDNA oxidative lesions. This notion is strengthened by the oxidative damage to mtDNA observed during the monoamine oxidase catalysis of biogenic amines [7], an effect explained by the diffusion of H_2O_2 into the matrix space. Hence, it may be surmised that H_2O_2 cleavage by DNA-bound metals may support a site-specific generation of $HO^{\bullet}$ leading to the vicinal accumulation of 8-HOdG.

The lower levels of 8-HOdG in GSH-depleted mitochondria and the higher levels in GSH-supplemented calf thymus DNA suggest a role for the non-protein thiol in the free radical steps leading to DNA oxidative damage. Evaluation of this effect requires consideration of: (a) the kinetic control inherent in the univalent reduction steps of O_2; (b) the catalyst role of metals either bound to ligands or in the bulk solution; and (c) the redox transitions of

GSH. A view integrating this reaction network is summarized in Fig. 1 and discussed below.

8-HOdG is usually considered a biomarker of nucleobase oxidative damage formed upon addition of $HO^{\bullet}$ onto desoxyguanosine (this occurs at diffusion controlled rates; $k > 10^9$ M^{-1} s^{-1}). Singlet oxygen attack on the base – yielding the same product– proceeds at substantially lower rates; $k = 5.2 \times 10^6$ M^{-1} s^{-1} [42]). In this experimental model, it is reasonable to assume that $HO^{\bullet}$ is responsible for mtDNA base oxidation; this assumption is based on the relationship between 8-HOdG and H_2O_2 levels [6,7] (the homolytic cleavage of the latter being an effective source of $HO^{\bullet}$ in a biological milieu) and the sensitivity of 8-HOdG accumulation to catalase.

Concerning the role of metals as catalysts in $HO^{\bullet}$ formation, it is feasible that DNA-Cu complexes may serve as effective ligands supporting a regiospecific mechanism for H_2O_2 cleavage and HO. production (Eq. 1. Copper is expected to bind tightly to a mononucleotide by way of both guanine–cytosine base pair regions [43] and phosphate sites [K(DNA+-$Cu^+ \Leftrightarrow$ DNA-Cu^+) = 10^9 M^{-1}] [44]. Although copper content in dialyzed calf thymus DNA was lower than that in undialyzed DNA, the unremoved (tightly bound) metals appear to be critical for nucleobase oxidation. Eq. 1 above proceeds slowly (k_1 = 1.2–1.3 M^{-1} s^{-1}) [44]; however, its rate-limiting character may be overcome by the rapid reduction of DNA-Cu^{2+} (Eq. 2)[1] and removal of $HO^{\bullet}$ in addition reactions that proceed at diffusion controlled rates.

$$\text{DNA} - \text{Cu}^{2+} + e^- \rightarrow \text{DNA} - \text{Cu}^{+} \qquad (2)$$

Because of the enhancing effect of superoxide dismutase on 8-HOdG accumulation in the presence of GSH, it is unlikely that $O_2^{\bullet -}$ serves as the sole electron donor for Eq. 2. This effect of superoxide dismutase may be explained by the rapid removal of $O_2^{\bullet -}$ generated during the univalent redox transitions of the thiol. The mechanism proposed in Fig. 1 is consistent with these findings inasmuch as GSH may serve as an electron donor for Eq. 2, thereby accounting for, on the one hand, the increase of 8-HOdG in its presence and, on the other hand, the further increase elicited by superoxide dismutase upon removal of $O_2^{\bullet -}$ generated during the decay of the disulfide anion radical ($GSSG^{\bullet -}$) (Fig. 1). That mitochondrial matrix GSH is not the only electron donor for Eq. 2 may be inferred from the partial (36–50%) protection against DNA oxidative damage in conditions of almost total (95%) GSH depletion.

The contribution of mitochondrial GSH to the accumulation of 8-HOdG in mtDNA reported here raises important questions concerning its biological significance. The susceptibility of mtDNA to oxidative damage has been ascribed to the lack of protective histones and the lack of DNA repair activity [9]. This and the constant exposure to oxidants generated by mitochondria may account for the high levels of 8-HOdG in mtDNA. Based on the results described in this study, the high levels of GSH in mitochondria appear to also play a determinant role in the process leading to an elevation of 8-HOdG. Thus, it may be concluded that the high oxidation rate of mtDNA is the result of a combination of factors: the properties of DNA and the oxidizing and reducing environment in mitochondria.

[1] Calculated rate for the reaction of $O_2^{\bullet -}$ with DNA-C^{2+} $\sim 10^8$ M^{-1} s^{-1}, J. Butler, personal communication.

Acknowledgements

This research was supported by Grant HL53467 from NIH. The authors thank Henry J. Forman for useful discussions.

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