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ARTICLE

Ferrous ion-induced strand breaks in the DNA plasmid pBR322 are not mediated by hydrogen peroxide

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Abstract Ferrous ion-induced generation of single and multiple strand breaks in the DNA plasmid pBR322 induces the formation of two new plasmid forms with altered electrophoretic mobility. The yield of these plasmid forms, the circular relaxed and the linear forms, depended on the applied Fe^{2+} concentration. This property was independent of the presence of hydrogen peroxide in the incubation mixture indicating the lack of Fenton chemistry to explain the DNA degradation. The removal of dioxygen or the presence of superoxide dismutase diminished partially the yield of ferrous ion-induced DNA plasmid degradation, while catalase was without any effect. Autoxidation of divalent iron as followed by the formation of a coloured iron-phenanthroline complex was enhanced in a concentration-dependent manner by phosphate and bicarbonate and very efficiently using a mixture of 0.15 M NaCl, 1.2 mM phosphate, 23.8 mM bicarbonate, pH 7.4, that concentrations correspond closely to the intracellular values of buffer components. Thus, the formation of a yet unknown reactive species from Fe^{2+}, and dioxygen, that is complexed to buffer components especially phosphate and its contribution in DNA plasmid degradation is more likely than the often cited formation of hydroxyl radicals in result of the Fenton reaction from Fe^{2+} and hydrogen peroxide.

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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Abbreviations

DNA	Desoxyribonucleic acid
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
UV	Ultraviolet

Introduction

A tight balance exists between degradation and repair of DNA in living cells. Attacks of free radicals and oxidants might cause modification of structural elements of DNA and induce single and multiple breaks of DNA strands (Halliwell and Aruoma 1991; von Sonntag 1994). Degradation of biological material is enhanced under conditions of oxidative stress and assumed to be a reason for changes in cell properties and metabolism under certain pathological conditions.

Free metal ions such as Fe^{2+} or Cu^{+} are important catalysts for oxidative damaging reactions in DNA and other biomolecules (Aruoma et al. 1991; Keyer and Imlay 1996; Kasprzak 2002). Iron and copper metabolism is strictly controlled in cells in order to avoid an increase of any free metal ions (Crichton and Ward 1998; Pomka 1999). Enhanced concentration of iron in the so-called ''labile iron pool'' is observed under stress situations and related to oxidation of biological material (Kakhlon and Cabantchik 2002; Kruszewski 2003).

It has been widely accepted that the oxidation of Fe^{2+} by hydrogen peroxide to Fe^{3+} is accompanied by the formation of highly reactive hydroxyl radicals according to $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$.

This reaction is known as the Fenton reaction. In a similar way, Cu^{+} promotes also the formation of hydroxyl radicals (Gunther et al. 1995). This reactive species is able to react nearly diffusion-controlled with a broad variety of biological substrates with the result that these substrates were oxidised and hydroxylised (von Sonntag 1985). Although the Fenton reaction has been firstly described for more than 100 years, mechanistic details are not well understood. It remains unsolved whether the often-cited hydroxyl radicals or excited iron complexes such as ferryl or perferryl compounds are the reactive products of this reaction (Rush and Koppenol 1986; Halliwell and Gutteridge 1988; Goldstein et al. 1993; Koppenol 1993; Wardman and Candeias 1996).

Some doubt arose about the significance of the Fenton reaction in damaging of biological material under stress situations several years ago. Free ferrous ions are slightly bound to biological buffer components such as phosphate or bicarbonate. Such complexes are catalytically active in hydroxylation of aromatic molecules (Miller et al. 1990; Biaglow and Kachur 1997). This incorporation of hydroxyl groups was also observed in the absence of hydrogen peroxide, suggesting a mechanism where apparently activated iron-dioxygen complexes were involved. Autoxidation of ferrous ions in the presence of phosphate is another mechanism that competes successfully with the Fenton chemistry in induction of deleterious free radical-mediated biological oxidations (Qian and Buettner 1999). The radical product of this reaction is not identical to the hydroxyl radical as shown by EPR probes (Reinke et al. 1994). The ferrous iron-dioxygen system has a high ability to cause free radicals that are able to hydroxylate salicylate (Urbanski and Beresewicz 2000). Strong evidence against the significance of the Fenton reaction in biological damaging came also from kinetic considerations of physiological concentrations of potential reactants and second order reaction rate constants of Fe^{2+}-driven reactions (Saran et al. 2000). These data made a role of the Fenton reaction in biological degradation highly unrealistic. The presence of free iron ions is quite sufficient for damaging biological substrates.

In order to prove this hypothesis, ferrous iron-induced generation of single and multiple strand breaks in the DNA plasmid pBR322 was investigated in the presence and absence of hydrogen peroxide. Due to strand breaks, the configuration of supercoiled DNA plasmid is changed and two new plasmid forms appear with altered electrophoretic mobilities. Our data show that the presence of ferrous ions and dioxygen is quite sufficient without the need of additional hydrogen peroxide to induce strand breaks in DNA. A binding of ferrous ions to buffer components such as phosphate and bicarbonate favours this iron-mediated DNA degradation.

Materials and methods

Materials

The DNA plasmid pBR322 from *E. coli* (catalogue number D 4904) was purchased from Sigma-Aldrich (Taufkirchen, Germany) as a stock solution (980 μg/ml) in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0. Topoisomerase 1 from human placenta, endonuclease *Bam*HI, superoxide dismutase from bovine erythrocytes, catalase from bovine liver, ethidium bromide, and desferoxamine were also applied from this company. Ferrous chloride and 1,10-phenanthroline were obtained from Riedel-de Haën (Taufkirchen, Germany). All other chemicals were from Fluka (Taufkirchen, Germany).

Stock solutions of $FeCl_2$ and $FeCl_3$ in deionised distilled water were prepared immediately prior use. The concentration of aqueous solutions of hydrogen peroxide was determined at 230 nm using $\epsilon_{230} = 74\ M^{-1}\ cm^{-1}$ (Beers and Sizer 1952).

Conformation standards

To get control samples for different plasmid conformations (Rice-Evans et al. 1991), the plasmid (25 μg/ml) was treated either with topoisomerase 1 (1 unit) or with the endonuclease *Bam*HI (5 units) for 15 min at 37°C. After incubation the reaction was stopped adding a half volume of a cocktail of 1% SDS, 50% (w/v) saccharose, and 0.1% bromophenol blue. Topoisomerase converts the plasmid into the circular relaxed form, whereas the treatment with endonuclease yields the linear form of the plasmid.

Incubation of the DNA plasmid pBR322 with iron ions

The plasmid pBR322 was diluted with 0.15 M NaCl, 1.2 mM phosphate, 23.8 mM bicarbonate, pH 7.4 or other buffer components to a final concentration of 25 μg/ml. The plasmid solution was incubated with

ferrous chloride in a final concentration ranging from 1.25 μM to 1 mM for 15 min at 37°C. The incubation was stopped adding a half volume of a cocktail of 1% EDTA, 50% (w/v) saccharose, and 0.1% bromophenol blue. In an other series of experiments, an equimolar mixture of hydrogen peroxide and $FeCl_2$ was incubated together with the plasmid. In these cases, hydrogen peroxide was always added first to the plasmid followed by $FeCl_2$.

Agarose gel-electrophoresis

All plasmid samples and controls were run on a 1% agarose gel containing 0.5 μg/ml ethidium bromide applying 74 V for 90 min in a small electrophoresis chamber (Mini-Sub® Cell-GT, Bio-Rad Laboratories, USA). Ten microlitres of a plasmid sample were applied on each lane. After running the samples were photographed under UV illumination at 312 nm by means of the Kodak DS electrophoresis documentation and analysis system 120. Quantitative assessment of track intensities was obtained using an Alpha imaging system Flexi 7070, Biostep Labor- and Systemtechnik GmbH, Johnsdorf, Germany.

Autoxidation of ferrous ions

A diluted aqueous solution of ferrous chloride is stable for several hours. However, ferrous ions are rapidly autoxidised in solutions containing bicarbonate and/or phosphate. The disappearance of Fe^{2+} has been followed in dependence on the concentration of buffer components with 1,10-phenanthroline that forms a coloured complex with Fe^{2+}. This ferrous ion chelator was added after different incubation times (ranging from 20 to 600 s) of Fe^{2+} with buffer components. The ferrous ion concentration was calculated from the extinction of the coloured ferrous ion-phenanthroline complex. Its molar extinction coefficient is equal to ϵ_{510} = 10,931 M^{-1} cm^{-1} (Yegorov et al. 1993).

Results

Hydrogen peroxide is not necessary in Fe^{2+}-mediated DNA degradation

The untreated plasmid pBR322 yields two bands in agarose gel electrophoresis, whereby about 70–75% correspond to the unperturbed supercoiled form without any single strand breaks (Fig. 1). The other 25–30% of this plasmid comprising the circular relaxed form are characterised by a reduced electrophoretic mobility. Topomerase I transforms the overwhelming part of the supercoiled fraction into the circular relaxed form. A third form, the linear form, with an intermediate electrophoretic mobility can be found applying endonuclease *Bam*HI that induces multiple strand breaks. Thus, the appearance of single strand and more complex multiple strand breaks in plasmid DNA can easily distinguished.

The plasmid pBR322 dissolved in a mixture of 0.15 M NaCl, 23.8 mM bicarbonate, 1.2 mM phosphate, pH 7.4 was incubated with ferrous ions in the concentration range from 1.25 to 1,000 μM for 15 min at 37°C in the presence or absence of hydrogen peroxide (Fig. 2). In experiments with hydrogen peroxide, a 1:1 concentration ratio between hydrogen peroxide and Fe^{2+} was always used. In both sets of experiments, the yield of the supercoiled fraction disappeared continuously at iron concentrations higher than 5 μM with increasing ferrous ion concentration, while a permanent increase in the fraction of the circular relaxed form was observed (Figs. 2, 3). No effects were found at Fe^{2+} concentrations of 5 μM and lower.

The linear plasmid form was found with increasing yield with increasing iron concentration only at Fe^{2+} concentrations higher than 100 μM. In case of the application of hydrogen peroxide, the formation of the linear form was slightly more pronounced in this concentration range. This was the only difference caused by the presence of hydrogen peroxide. Control experiments revealed that the sole addition of hydrogen

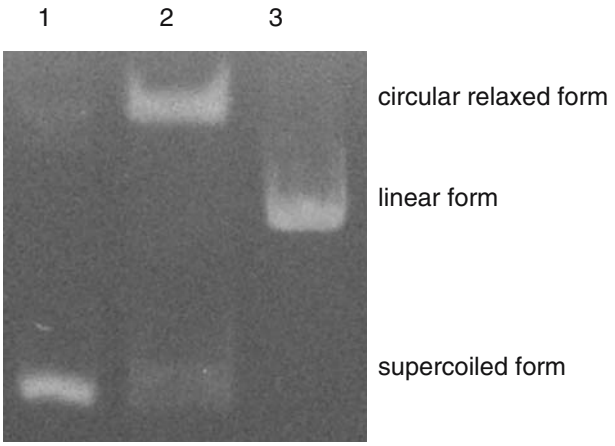


Fig. 1 Electropherogram of the plasmid pBR322 treated with topoisomerase I or endonuclease *Bam*HI. The plasmid was diluted with 0.15 M NaCl, 1.2 mM phosphate, 23.8 mM bicarbonate, pH 7.4 to a final concentration of 25 μg/ml. It was treated with topoisomerase 1 (1 unit, *lane 2*) or with the endonuclease *Bam*HI (5 units, *lane 3*) for 15 min at 37°C. *Lane 1* shows the untreated plasmid

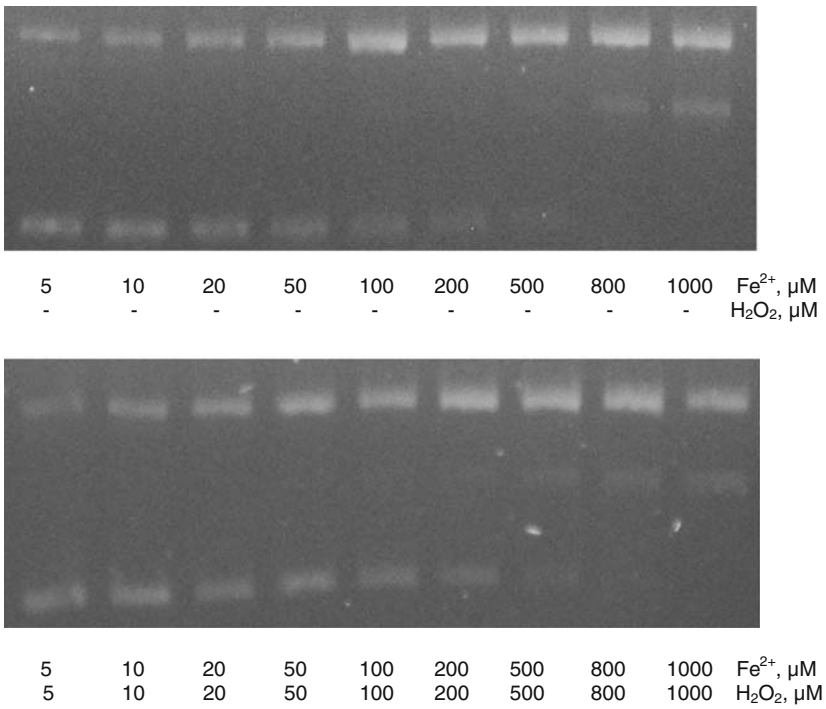


Fig. 2 Effects of Fe^{2+} on the electrophoretic mobility of the plasmid pBR322 in the absence (*upper part*) and presence (*lower part*) of hydrogen peroxide. The plasmid (25 μg/ml) was treated with $FeCl_2$ and hydrogen peroxide for 15 min at 37°C. Representative data of four independent experiments are shown

peroxide did not cause any degradation of the DNA plasmid (data not shown).

Thus, Fe^{2+} is an important component for the fragmentation of the DNA plasmid pBR322, whereas hydrogen peroxide was not necessary to promote this effect. The application of sole ferric ions up to concentration of 1 mM did not cause any effects on the plasmid structure (data not shown).

The high dependence of plasmid degradation on Fe^{2+} is also supported by the fact that chelators of Fe^{2+} such as EDTA, 1,10-phenanthroline and diethylenetriaminepentaacetic acid (DTPA) efficiently inhibited the plasmid degradation (data not shown). The strong action of EDTA is apparently responsible for the ineffectiveness of very low Fe^{2+}-concentrations to mediate the plasmid degradation. The diluted DNA plasmid pBR322 sample contains approximately 20 μM EDTA.

Effects of phosphate and bicarbonate on plasmid degradation

The presence of the buffer components phosphate and bicarbonate was also necessary for the appearance of strand breaks. In all experiments described so far, a mixture of 0.15 M NaCl, 1.2 mM phosphate and 23.8 mM bicarbonate has been used. This composition of active buffer components resembles closely to the medium composition in cells. Using the same ratio between phosphate and bicarbonate, there was no alteration in the appearance of the circular relaxed plasmid form at 100 μM Fe^{2+} by doubling or halving the buffer capacity (Fig. 4). Also the same result was found using 50 mM bicarbonate as buffer component. However, a much higher plasmid degradation with formation of the linear form occurred in 50 mM phosphate at 100 μM Fe^{2+}.

Autoxidation of ferrous ions by phosphate and/or bicarbonate

The disappearance of Fe^{2+} in phosphate and bicarbonate containing solutions due to autoxidation was followed by 1,10-phenanthroline. Both phosphate and bicarbonate enhanced the rate of ferrous ion disappearance, whereby phosphate exhibited a more pronounced effect than bicarbonate at the same concentration (Fig. 5). The use of a phosphate/bicarbonate mixture (0.15 M NaCl, 1.2 mM phosphate, 23.8 mM bicarbonate, pH 7.4) has a much higher effect than the sole use of both single components underscoring a synergistic effect of both phosphate and bicarbonate.

Role of further components in iron-mediated plasmid degradation

Selected experiments have been provided to evaluate further requirements necessary to induce single and multiple strand breaks in the DNA plasmid pBR322. The addition of catalase at 1,000 U/ml did not change

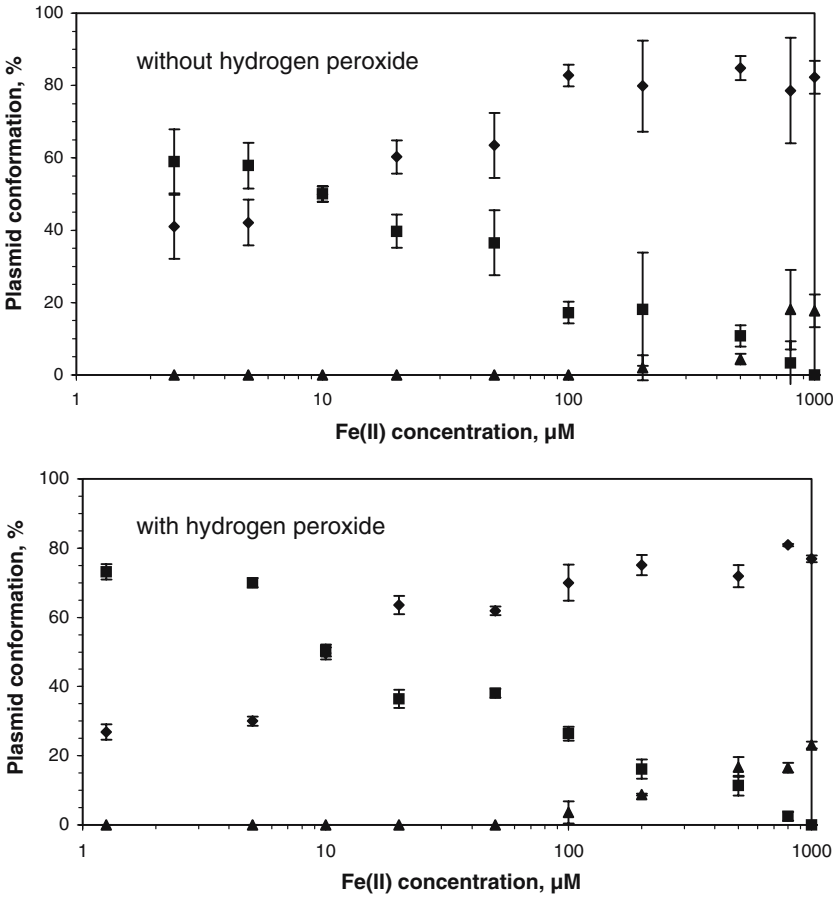
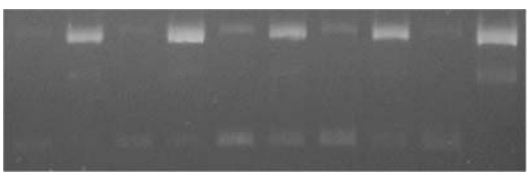


Fig. 3 Percentages of the supercoiled (*filled square*), circular relaxed (*filled diamond*), and linear (*filled triangle*) plasmid forms in dependence on the Fe^{2+} concentration in the absence (*upper part*) and presence (*lower part*) of hydrogen peroxide. In experiments with hydrogen peroxide, a 1:1 concentration ratio between hydrogen peroxide and Fe^{2+} was always used. All other experimental conditions are given as in Fig. 2. Means and standard deviations of three independent experiments are shown

the plasmid structure in comparison to the control (Fig. 6). This is a further evidence that free hydrogen peroxide was not involved in plasmid fragmentation. The addition of superoxide dismutase at 500 U/ml inhibited partially the formation of the circular relaxed and linear plasmid forms at 100 and 1,000 μM $FeCl_2$. Especially using the later ferrous ion concentration, the original unperturbed supercoiled plasmid form was still seen in the electropherogram.



-	0.1	-	0.1	-	0.1	-	0.1	-	0.1	$FeCl_2$
150	150	150	150	150	150	150	150	150	150	NaCl
11.9	11.9	23.8	23.8	47.6	47.6	50	50	-	-	HCO_3^-
0.6	0.6	1.2	1.2	2.4	2.4	-	-	50	50	Phosphate

Fig. 4 Effects of phosphate and bicarbonate concentrations on the electrophoretic mobility of the plasmid forms after addition of $FeCl_2$ (100 μM, final concentration). All reactant concentrations are indicated in the figure. All other experimental conditions are used as in Fig. 2

Incubation medium was also treated with nitrogen gas for 15 min in order to reduce the dioxygen tension. In this case, Fe^{2+} lost partially its ability to induce the

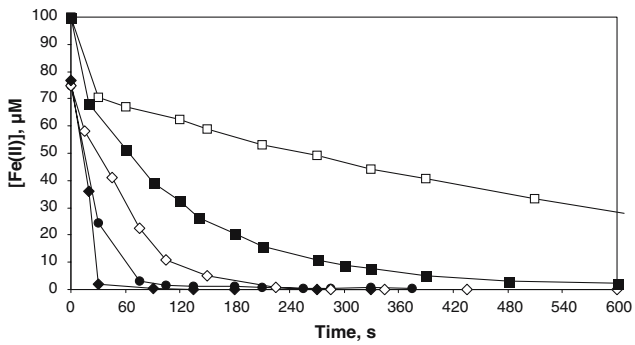


Fig. 5 Autoxidation of Fe^{2+} as a function of incubation time and phosphate and bicarbonate concentration. An aqueous solution of freshly prepared $FeCl_2$ (100 μM, final concentration) was added at the time point 0 s to 10 mM phosphate (*open diamond*), 100 mM phosphate (*filled diamond*), 10 mM bicarbonate (*open square*), 100 mM bicarbonate (*filled square*), or a mixture of 1.2 mM phosphate and 23.8 mM bicarbonate (*filled circle*). Then, 1,10-phenanthroline (500 μM, final concentration) was added after different time intervals (ranging from 20 to 600 s) in order to determine the concentration of the remaining ferrous ions. Representative data of four independent experimental series are shown

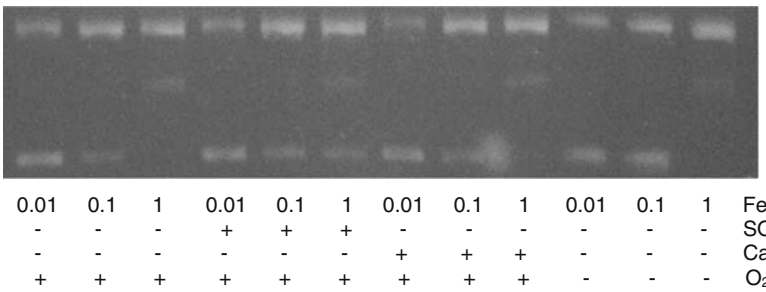


Fig. 6 Effects of superoxide dismutase (500 U/mg), catalase (1,000 U/mg) and dioxygen on the Fe^{2+}-mediated degradation of the plasmid pBR322. All other experimental conditions are used as in Fig. 2. To produce a dioxygen-depleted medium, nitrogen gas was bubbled for 15 min through the plasmid solution. Representative data from three independent experiments are shown

fragmentation of the DNA plasmid (Fig. 6). There is a lower yield of the circular relaxed plasmid form using 100 μM Fe^{2+} and only a very small formation of the linear form at 1,000 μM Fe^{2+}.

Discussion

Oxidative damage of DNA by metal ions leads to numerous modifications of the molecular structure including base damage, cross-linking reactions, strand scissions and depurination (Kasprzak 2002). Here, we focused our attention on the induction of DNA strand breaks by Fe(II) and the potential role of hydrogen peroxide in this damage. A mixture of 0.15 M NaCl, 1.2 mM phosphate, 23.8 mM bicarbonate, pH 7.4 has been selected in this study because the components of this medium occur in similar concentrations in cell cytoplasm. These components are contained at the same concentration in the Krebs–Henseleit buffer, that is widely used as physiological medium to store cells and tissues. Krebs–Henseleit buffer contains additionally glucose, KCl, $CaCl_2$, and $MgSO_4$.

Ferrous iron added to a mixture of 0.15 M NaCl, 1.2 mM phosphate, 23.8 mM bicarbonate, pH 7.4 promoted in a concentration-dependent manner the degradation of the DNA plasmid pBR322 with the appearance of single and multiple strand breaks. Hydrogen peroxide was not necessary to induce this effect. Thus, the formation of deleterious free radicals derived from the Fenton reaction can be excluded as a main reason for DNA degradation under our experimental conditions.

As the plasmid degradation was attenuated under nitrogen atmosphere, the formation of free radicals derived from ferrous ion–dioxygen complexes is more likely. This pathway was also favoured in iron- and copper-mediated biological degradation by other authors (Reinke et al. 1994; Qian and Buettner 1999; Urbanski and Beresewicz 2000). A chelation of ferrous iron by buffer components especially by phosphate enhanced markedly the autoxidation rate of Fe^{2+} as well as the appearance of DNA cleavage. In this way, a reactive species of unknown nature complexed to buffer components is formed in our experiments. Saran and coworkers postulated the formation of ''crypto-OH radicals'' as reactive species in the result of interaction of Fe^{2+} with phosphate and/or bicarbonate and calculated a second order reaction rate constant of 1,700 M^{-1} s^{-1} for this process (Saran et al. 2000). From reactant concentrations under stress conditions, these authors concluded that the Fenton reaction has no importance under in vivo conditions.

The exact nature of oxidants in Fe^{2+}-mediated oxidations of biological material is still a matter of debate. Necessary components for the formation of the active catalytic complex are Fe^{2+}, dioxygen, and weak chelators such as phosphate and bicarbonate. In as much Fe^{3+} is additionally involved is unsolved. Fe^{3+} alone did not induce the plasmid degradation. However, the presence of Fe^{3+} in buffer solutions enhanced markedly the appearance of Fe^{2+}-induced strand breaks in the DNA plasmid. Fe^{2+}–dioxygen–Fe^{3+} complexes have been proposed by several authors to promote oxidative damage of biomaterials (Miller and Aust 1981, 1987; Ryan and Aust 1992).

Free hydrogen peroxide did not contribute to the DNA plasmid degradation in our experiments as catalase was without any effect. On the other hand, superoxide anion radicals are involved as intermediate in the plasmid degradation. Superoxide dismutase inhibited markedly the plasmid degradation in our experiments. Qian and Buettner (1999) found also a partial inhibition by SOD and combined SOD and catalase of the formation of oxidation products in Fe(II)-mediated oxidation of dimethylsulfoxide,

ethanol, and glucoce. Superoxide anion radicals are formed in the result of autoxidation of Fe^{2+}. However, it cannot be ruled out that around the chelated iron a caged iron-dioxygen complex is formed where other reactive oxygen species are trapped and involved in the oxidation process.

Phosphate accelerates the autoxidation of Fe^{2+} more drastically than bicarbonate as followed with 1,10-phenanthroline (see Fig. 5). Phosphate showed also a higher activity in plasmid degradation than bicarbonate in sole application at the same buffer concentration of 50 μM (Fig. 4). In cell cytoplasma, bicarbonate is about 20-fold more concentrated than phosphate. Apparently, both buffer components act synergistically in the ferrous ion-mediated DNA plasmid oxidation, whereby phosphate is more involved in chelation and autoxidation. New data from other groups support the role of bicarbonate-derived oxidants in oxidation of biological targets. As has been recently shown the oxidation of bicarbonate leads the reactive oxidants such as bicarbonate anion radical (Augusto et al. 2002) and peroxymonocarbonate (Richardson et al. 2003). Moreover, bicarbonate favours the oxidation of thiol groups in amino acids and bovine serum albumin induced by peroxynitrite (Bonini and Augusto 2001) or in hydrogen peroxide-driven oxidations (Bonini et al. 2004).

Although mechanistic details remain unknown in Fe^{2+}-mediated degradation of the DNA plasmid pBR322, the Fenton reaction as source for the reactive species can be ruled out. More likely, a reactive species is produced due to autoxidation of Fe^{2+} enhanced by buffer components in combination with bicarbonate oxidation.

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