GENETIC DELETERIOUS EFFECTS OF SINGLET OXYGEN

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Singlet molecular oxygen ($^1O_2$) has been shown to be one of the most reactive oxygen species, causing damage to the genetic material. Several DNA modifications were identified as products of the selective oxidation of guanine residues by $^1O_2$, including 8-oxo-7-hydrodeoxyguanosine as, probably, the major lesion. The available data point to an important role of the induced DNA lesions in cytotoxicity and loss of biological plasmid activity by $^1O_2$. This excited molecule has also been shown to be highly mutagenic in both pro and eukaryotic cells, and the mutations (mostly G:C to T:A transversions) are targeted to guanine modified bases. Moreover, there is a variety of DNA repair genes involved on the processing of $^1O_2$-induced DNA lesions, suggesting that these cellular defenses may be related to the *in vivo* $^1O_2$ deleterious role.

Keywords: singlet oxygen; DNA lesions; mutagenicity; DNA repair.

INTRODUCTION

The generation of electronically excited molecular oxygen (singlet oxygen, $^1O_2$) has been shown to occur in several biological systems, such as photooxidation of different biological compounds and xenobiotics, lipid peroxidation and also enzymatic reactions. As other activated oxygen species, including the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$), $^1O_2$ may be implicated in the initial events leading to several human pathological processes like arthritis, lung oxidant injury, skin photosensitivity, erythropoietic porphyria, artherosclerosis, cancer and aging. The basis for such involvement is the high reactivity of $^1O_2$, which is a strong electrophile oxidizing biological molecules with high electronic density. It has been found to be capable of damaging several biological targets including lipids, proteins and nucleic acids. However, unravelling the role of $^1O_2$ in biological systems has been hampered by the difficulties to obtain $^1O_2$ free from other reactive species. Thus, the evaluation of available literature gives partly conflicting results regarding the significance of $^1O_2$-hazards in biological systems, such as DNA damage and mutagenesis. In this paper, we present a review on the data concerning the main consequences of $^1O_2$ interaction with genetic material. This work has been greatly benefitted from recent reviews on the genotoxicity of $^1O_2$, notably those by Epe[1] and Piette[2].

PHYSICAL ASPECTS OF SINGLET OXYGEN

The molecular oxygen exhibit the unusual paramagnetic behavior, having the ground triplet state, $^3\Sigma_g^-$. As a consequence, the direct oxygen reduction by two electrons is spin-forbidden. The activation of oxygen to electronically excited states with antiparalell spins requires an overcoming of spin restriction. There are two excited states: the first in which both electrons occupy the same orbital, $^1\Delta_g$, and the second in which the electrons have opposed spins, but occupy different orbitals, $^1\Sigma_g^+$ (figure 1). The second singlet state is extremely short lived, being rapidly (1.10^{-11} s) deactivated to the $^1\Delta_g$ state. It is, therefore of minor interest in biological systems. Thus, the term 'singlet oxygen' or $^1O_2$ usually stands for $^1\Delta_g\ O_2$.

The infrared electronic transition of $^1O_2$ to ground state (1270 nm) is highly forbidden, so that it has a lifetime of 45

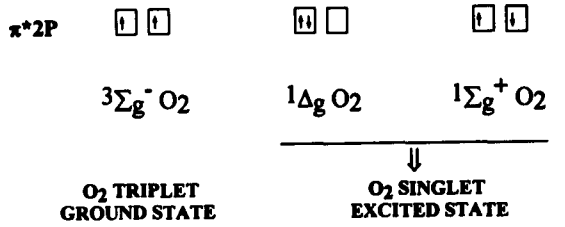

Figure 1. Ground and excited states of molecular oxygen.

min calculated for zero pressure[3]. In water the $^1O_2$ lifetime is considerably reduced to the range of 4-50 μs, but diffusion of this molecule in biological systems is still possible within a radius estimated to be up to 100 Å[4,5]. The lifetime of $^1O_2$ is markedly increased when hydrogen atoms in the solvent are replaced by deuterium[6]. Thus, the use of deuterated water (D_2O), increasing $^1O_2$ lifetime 15 to 18 fold, has become universal to characterize the presence of $^1O_2$ and its action.

SINGLET OXYGEN IN BIOLOGICAL SYSTEMS

Photodynamic effects, mediated by light, O_2, and a photosensitizing dye, were the first example of the interaction of $^1O_2$ with biological systems[7]. The implications of photosensitization have been reviewed by many investigators[8,9]. Only some of those will be commented here. Furocumarin derivatives (psoralens) were used as photosensitizers during UV treatment of psoriasis, and other types of skin diseases; $^1O_2$ production has been demonstrated from irradiated psoralens[10]. Porphyrins are also $^1O_2$-generating photosensitizers[11]. The widespread use of derivatives of hematoporphyrins as photosensitizing agents in the treatment of different types of tumors, as they accumulate in proliferating cells, appears to be associated with the ability of these porphyrins to generate $^1O_2$ when illuminated *in situ*[12]. Tetracyclins, used as antibiotics, generate $^1O_2$, as shown by Hasan and Khan[13]. It was suggested that $^1O_2$ formation may explain side effects of tetracyclins such as cutaneous phototoxicity.

Damage can also occur to a variety of systems when sensitizers are generated *in vivo*. A classic example of endogenous formation of a photosensitizer is the accumulation of

protoporphyrin IX in carriers of erythropoietic porphyria, an inborn defect in the heme biosynthetic pathway. The cataract that appears in elderly individuals has been attributed to the photosensitized formation of tryptophan derivatives, which in turn generate $^1O_2$ leading to cross-linking of lens proteins[14]. This disease seems to be linked both to formation of glycosylated proteins and autoxidations leading to active oxygen species.

Enzymatic formation of $^1O_2$ has been shown to occur in several cases. Examples are those catalyzed by dioxygenases[15], tryptophan pyrrolase[16] and lipoxygenases[17]. The generation of $^1O_2$ by polymorphonuclear neutrophils or by myeloperoxidase during bactericidal action as well as during lactoperoxidase activity has been suggested. Kanofsky[18,19] and Khan[20] provided evidence that microbactericidal activity is related to $^1O_2$ generation as they measured the 1270 nm chemiluminescence from lactoperoxidase and chloroperoxidase supplemented with H_2O_2 and halide ions. Recently, Steinbeck *et al.*[21] demonstrated that $^1O_2$ is produced by neutrophils during the process of phagocytosis, by using glass beads coated with a specific chemical trap for $^1O_2$ (9,10-diphenylanthracene).

The participation of $^1O_2$ during the biosynthesis of prostaglandins was first suggested by Samuelsson[22]. Cadenas *et al.*[23] obtained evidence for $^1O_2$ participation in the metabolism of arachidonic acid by prostaglandin-endoperoxidase synthase through the chemiluminescence spectrum which shows maximum ligth intensity at about 634 and 703 nm. An almost identical spectrum was obtained using isolated cytochrome P_{450} or microsomal fractions supplemented with hydroperoxide or iodosobenzene as an oxene donnor[24].

Regarding biological systems as subcellular fractions, cells and intact tissues, chemiluminescence has been measured during conditions of oxidative stress. Lipid peroxidation in microsomal fractions initiated by hydroperoxides or iron/ascorbate, as well as lipid peroxidation of isolated hepatocytes have been studied extensively by Cadenas *et al.*[25]. The available evidence points to $^1O_2$ formation *via* the Russell mechanism[26].

In figure 2, a scheme illustrates several biological systems that generate $^1O_2$ as well as their possible consequences.

IN VITRO DNA DAMAGE

In general, most of the experiments designed to elucidate the interaction of $^1O_2$ with nucleic acids are performed *in vitro*. This provides efficient manners to determine and control the action of $^1O_2$. As a consequence, several DNA lesions were identified and they are now being investigated in order to help the understanding of the *in vivo* role of $^1O_2$ on genotoxicity and mutagenicity.

Reactivity with free nucleosides in solution

The specific reactivity of $^1O_2$ with guanine was first reported in the early sixties. Trying to understand the photodynamic action of methylene blue on nucleic acids, Simon and Van Vunakis[27,28] observed that, in the presence of oxygen, the guanine residues were preferentially attacked, leading to the loss of ultraviolet (UV) absorbance due to the guanine decomposition. Little or no effect was observed for the other DNA or RNA nucleotides. It should be noted that there are two types of sensitized photooxidations[29,30]. In the type I reaction, the triplet sensitizer reacts directly with substrate, usually originating other radicals that may attack DNA and its components. In the type II reaction, the triplet sensitizer interacts with oxygen to generate $^1O_2$. Therefore, the specific action of $^1O_2$ in these systems must be examined with caution. The deoxyguanosine (dG) decomposition by photodynamic action was further examined by comparing several different dyes, and the data showed that it correlates well with the production of $^1O_2$ in each case[31]. The products of the photooxidation of dG in solution sensitized by several dyes were studied in detail by Cadet *et al*[32]. By employing specific $^1O_2$ quenchers and D_2O solvent, they concluded that the reaction proceeds mainly by the intermediate formation of $^1O_2$. Among the photoproducts, they identified two diastereoisomers of the 4-hydroxy-8-oxo-deoxyguanosine derivative, and the cyanuric acid derivative (figure 3). Lee and Rodgers[33] have analysed the reactivity of $^1O_2$, generated by photoactivation of rose bengal, with all four nucleotides components of DNA. Only guanine residues showed a significant reaction with $^1O_2$. Recently, Devasagayam *et al.*[34] have found that $^1O_2$ oxidizes guanine nucleosides in solution leading to the formation of 8-oxo-7-hydrodeoxyguanosine (abbreviated 8-oxodG, figure 3). The corresponding 8-oxo derivative is not formed from deoxyadenosine. In this work, the $^1O_2$ was generated by the thermodissociation (at 37°C) of the water-solu-ble endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate ($NDPO_2$), a method through which $^1O_2$ can be obtained in an easy and simple way without reactive intermediates or byproducts[35].

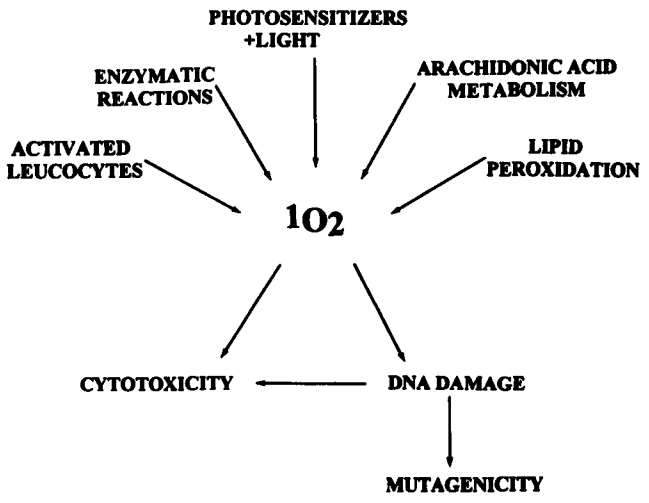

Figure 2. Generation and effects of $^1O_2$ in biological systems.

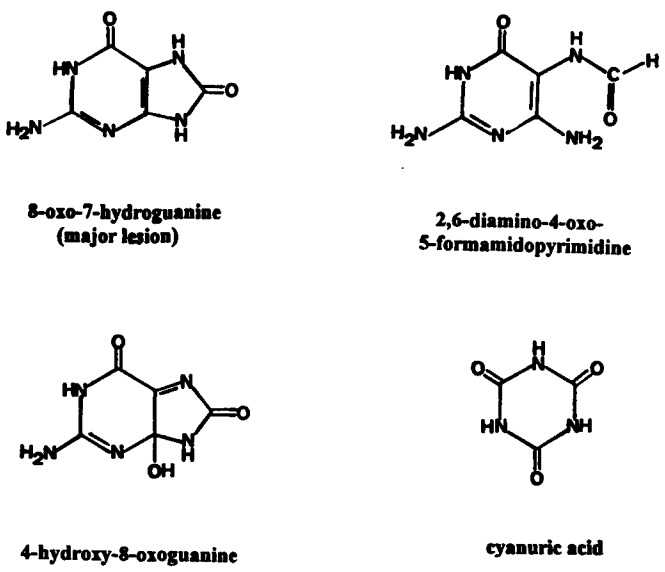

Figure 3. Damaged guanine residues induced by $^1O_2$.

Reactivity with DNA

Several evidences also support the specific reactivity of $^1O_2$ with guanine within the DNA molecule. Photosensitization by hematoporphyrin has been shown to cause selective degradation of the guanine moiety[36]. Exposure of DNA double helix to photosensitized methylene blue was found to generate modifications of guanine residues, that are detected by piperidine treatment, leading to chain cleavage at damaged position[37,38]. Sites sensitive to piperidine cleaved specifically at guanine residues were also observed as a result of treating single stranded DNA with hematoporphyrin or methylene blue plus light[39]. In that work, the use of ESR spectroscopy and specific $^1O_2$ traps strongly indicated that both photosensitizers react with guanine *via* a Type II mechanism (involving $^1O_2$).

Blazek *et al.*[40] have employed the separated-surface-sensitizer system[41] as a source of pure $^1O_2$ to demonstrate that this excited molecule yields piperidine- and alkali-labile sites in supercoiled plasmid DNA. These authors also detected single strand breaks in the phosphodiester DNA backbone as a consequence of $^1O_2$-treatment. Replacement of H_2O by D_2O in the treatment-buffer increases the DNA lesions yield, consistent with the participation of $^1O_2$ as an intermediate in the reaction. The breakage of DNA chain by $^1O_2$ was also observed by many different laboratories. Water-soluble porphyrins, in the presence of oxygen and light, were shown to induce single and double strand cleavage of plasmid DNA, and the inhibitory effect of azide anion (N_3^-) indicated the involvement of $^1O_2$ in the mechanism of DNA cleavage[42,43]. Di Mascio *et al.*[44] have used three different techniques to generate $^1O_2$ (microwave discharge, photosensitization of immobilized rose bengal and $NDPO_2$ thermodissociation) and showed the induction of single strand breaks on supercoiled bacteriophage and plasmid DNA. This was confirmed by $NDPO_2$-treatment of plasmid DNA[45,46]. Exposing an *E.coli*/mammalian cell shuttle vector (see below) to $^1O_2$, generated by thermodissociation of $NDPO_2$, Di Mascio *et al.*[47] observed the induction of single and double strand breaks on the DNA molecule. The double strand breaks were proposed to be a result of DNA breakage in guanine-rich sites. Breaks were also shown to occur after $NDPO_2$-treatment of single stranded DNA[48]. Interestingly, quantification of such breaks indicated that single stranded DNA structures are much more sensitive than double helix molecules. This can be explained by the increased accessibility of the guanine residues, the primary target of $^1O_2$, in single stranded DNA, when compared to the well arranged bases inside the double helix. Finally, Devasagayam *et al.*[34] located the DNA strand breaks (induced by $NDPO_2$-treatment) selectively at dG positions, supporting the idea that this base is the primary substrate to $^1O_2$ attack leading to breakage of the sugar-phosphate DNA backbone. In most of these works, the mediation of $^1O_2$ in DNA damage is supported by the enhancing effect caused by solvent deuteration. It should be mentioned that these reports conflict with the work presented by Nieuwint *et al.*[49] and Lafleur *et al.*[50], who were unable to detect DNA breaks on plasmid exposed to $^1O_2$. However, in these experiments the effective concentration of $NDPO_2$ employed was very low.

Other kinds of DNA damage were also identified in molecules exposed to $^1O_2$. Floyd *et al.*[51] have used HPLC and an electrochemical detector to demonstrate the production of 8-oxodG on calf thymus DNA exposed to methylene blue plus light. The yield of 8-oxodG may be approximately 2% of the guanine present on DNA. The enhancement effect of D_2O and the requirement of oxygen suggest that $^1O_2$ is the main intermediate in this reaction. Comparison on the amount of 8-oxodG and the number of DNA breaks induced by this treatment indicated that single strand breaks occur much less frequently than the modified guanine derivative[52]. Boiteux *et al.*[53], using Fpg protein (formamidopyrimidine-DNA glycosylase) as an enzyme probe and gas chromatography/mass spectrometry, reported the formation of 2,6-diamino-4-oxo-5-formamidopyrimidine (a guanine derivative with an open imidazole ring- FapyG, figure3) and 8-oxodG after exposing calf thymus DNA to photosensitized methylene blue. Adenine and pyrimidines were not significantly affected under the conditions used, and the amount of FapyG induced was approximately 20 fold lower than of 8-oxodG. Both compounds were proposed to be a result of oxidation of guanine residues by $^1O_2$.

DNA lesions that block DNA synthesis *in vitro* by *E.coli* DNA polymerase I (Klenow fragment) were detected by Piette and Moore[54] after exposure of single stranded ØX174 DNA molecules to proflavine and light. Chain termination was visualized in DNA sequencing gel and found to be at one nucleotide preceeding guanine residues. The frequency of these blocking lesions was inhibited by NaN_3 and enhanced by D_2O, suggesting that at least part of guanine modifications resulted from the action of $^1O_2$[55]. Recently, these data were confirmed for single stranded plasmid DNA treated with $NDPO_2$[56]. Many different DNA polymerases were employed and all had DNA synthesis interrupted close to guanine residues. Modified T7 phage (sequenase) and *Thermus aquaticus* DNA polymerases synthesized DNA fragments which terminated opposite dGs, while T4 phage DNA polymerase and avian myeloblast virus reverse transcriptase were blocked one nucleotide before dGs on the template. *E.coli* DNA polymerase I (Klenow fragment) was inhibited both at and before dGs. The number of lesions was quantified and, at 5 mM of $NDPO_2$, they were estimated to correspond approximately to 2% of guanines on the DNA and 20 times the amount of breaks on DNA treated with $NDPO_2$ in similar conditions. These values are significantly close to those reported for 8-oxodG induction in DNA damaged by methylene blue plus light[51,52] and led the authors to propose that at least part of the DNA synthesis blocking lesions was in fact 8-oxodG. This is supported by Shibutani *et al.*[57], who found that this modified guanine interrupts transiently DNA synthesis *in vitro*.

The data described above indicate a high selective reactivity of $^1O_2$ with guanine residues either as free nucleosides or in DNA. Several DNA lesions were identified until now, including DNA cleavage, alkali and piperidine-labile sites, 4-hydroxy-8-oxodG, cyanuric acid, FapyG and 8-oxodG. This last guanine modification, 8-oxodG, seems to be the main product of $^1O_2$ attack to DNA. The biological relevance of such damages is still being recognized, but they constitute blocks or at least transient interruptions to DNA polymerization.

DNA INACTIVATION AND CELLULAR GENOTOXICITY

As stated above, *in vitro* experiments allow one to control the direct reactivity of $^1O_2$ with DNA. Specific effects on this molecule can then be assigned, or not, to the action of $^1O_2$. Therefore, the use of DNA molecules as exogenous probes to search the consequences of this excited molecule on genetic material has been widely accepted. In general, plasmid or phage DNA is exposed to a source of $^1O_2$ and then introduced into the living cell, where the $^1O_2$-induced lesions are processed by the cell enzymatic machinery. The biological consequences of these damages can then be followed, and eventually the effects are correlated with any of the known DNA lesions identified (figure 4).

The first event that is characterized using exogenous DNA probes is their biological inactivation, that is loss of the ability to transform cells, upon $^1O_2$-treatment. For prokaryotic cells, this has been observed for ØX174 phage DNA[44,58], M13 phage DNA[44,59] and plasmid DNA[44,60]. Single stranded DNA

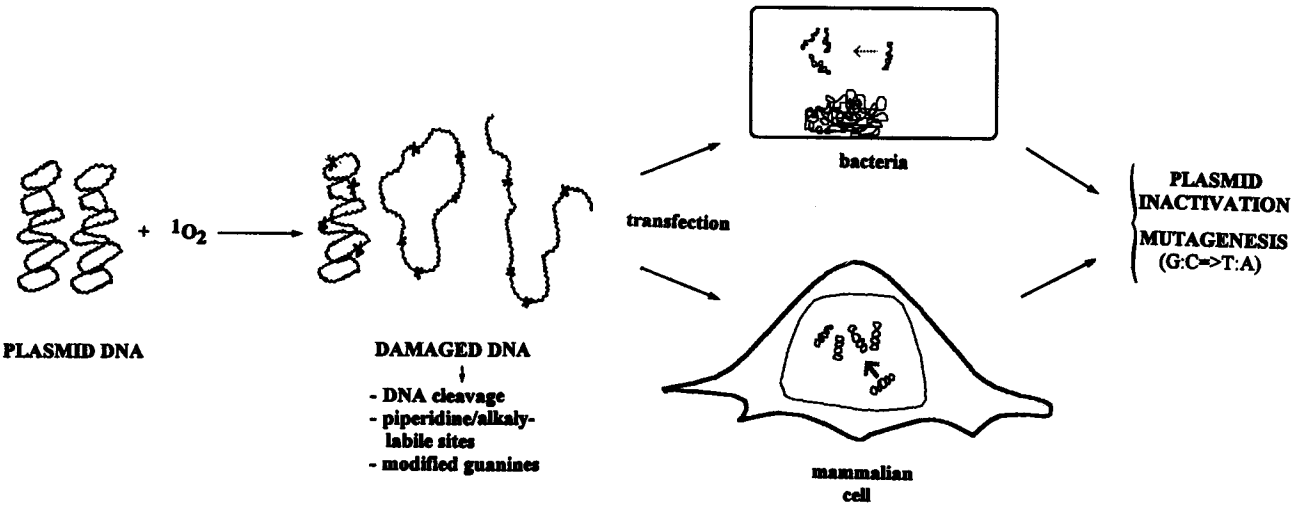

Figure 4. Use of exogenous DNA probes to investigate the biological effects of $^1O_2$.

probes were found to be inactivated more efficiently than double stranded DNA[44,50], correlating well with the higher amount of DNA lesions (single strand breaks) observed in single stranded DNA treated with $^1O_2$[48]. These experiments employed different techniques to generate $^1O_2$, normally in conditions that favors its activity (eg. D_2O), so that the loss of transforming activity is probably due to the oxidation of DNA by this excited molecule.

For eukaryotic cells, a similar approach was employed, having plasmid shuttle vectors as probes. The shuttle vectors used are hybrid plasmids containing pBR322 DNA sequences for replication and selection in *E.coli* and a portion of the primate virus SV40 for replication in monkey cells. As a consequence the vector replicate in both bacteria and mammalian cells[61]. In usual experiments, the vector is exposed *in vitro* to $^1O_2$ (generated by $NDPO_2$ thermodissociation) and introduced into monkey COS7 cell line, where the lesions can be repaired and replicated in the eukaryotic environment. The vectors are rescued back to *E.coli* where the colonies recovered reflect the active plasmids in the monkey cells. Opposite to what is observed for bacteria, double stranded shuttle vectors are not inactivated in mammalian cells except at very high doses of $^1O_2$[47]. These data are consistent with a very efficient DNA repair system for $^1O_2$-mediated DNA damages in mammalian cells[62]. Nevertheless, when similar experiments were performed using single stranded DNA shuttle vectors, high levels of plasmid inactivation upon $^1O_2$-exposure were observed[48]. Besides carrying more lesions than double stranded molecules, single stranded DNA also does not have a template strand for repair, so that the DNA damages are effective, leading to the loss of biological activity of this molecule.

The lesion(s) responsible for the DNA loss of transforming activity in pro or eukaryotic cells is(are) not known. The number of single strand breaks induced by $^1O_2$ paralells the biological inactivation of supercoiled DNA in bacteria[44,62], but the oxidized guanine residues probably also exert a role on the lethal effects of $^1O_2$ on DNA.

Although experiments involving treatment of whole cells with $^1O_2$ are more difficult to interpret, there are several lines of evidence that $^1O_2$ may cause cell death in bacteria, yeast or mammalian cells. $^1O_2$ has been reported to be the main mediator in the bactericidal action of human polymorphonuclear leukocytes[63] and of many photosensitizers[13,64,65,66,67]. The cytotoxicity for bacteria was also observed when the cells were treated by exogenous $^1O_2$[68,69] generated by the separated-surface-sensitizer method[41]. The photodynamic inactivation of yeast cells has been reported and $^1O_2$ is probably the major intermediate in this effect[64,70]. For mammalian cells, photosensitizers cause cell death in cultured Chinese hamster cells[71] and human fibroblasts[72]. Extracellular pure $^1O_2$ causes cytotoxic effects in human lymphocytes[73]. $^1O_2$ has also been implicated as intermediate in the direct inactivation of human fibroblasts by UVA (334 nm, 365 nm) and near-visible (405 nm) radiations[74]. Therefore, the idea that $^1O_2$ can generate cell damage that may lead to its inactivation seems to be a consensus. However, the cellular target responsible for cell death provoked by $^1O_2$ is not known. Different papers report genetic effects after cell treatment with photosensitizers which may diffuse into the cells. Alkali-labile sites, but no DNA strand breaks, were detected in chromosomal DNA of *E.coli* after cell exposure to hematoporphyrin and UVA (365 nm)[75]. The use of D_2O-containing buffer yields a higher number of alkali-labile sites, indicating that these lesions are induced by $^1O_2$. DNA strand breaks were detected in alkaline conditions in Chinese hamster cells exposed to photosensitized chloroaluminum phthalocyanine[71], and in human cells treated with hematoporphyrin and visible light[76] or tetra(3-hydroxyphenyl) porphyrin[72]. Epe *et al.*[77] found that DNA base modifications are induced in bacteria (*Salmonella typhimurium*) by treatment with methylene blue and light. The similarities of these DNA lesions in cellular and those observed in isolated DNA treated with the photosensitizer provide the best indication that, at least in these experiments, $^1O_2$ may react directly with DNA within the cell. Gene conversion induced by photosensitized acridine orange was observed in yeast and correlated with the action of $^1O_2$ as the major intermediate[70]. Sister chromatid exchanges were also observed in human cells exposed to hematoporphyrin and light[76]. Moreover, evidences for mutagenesis in $^1O_2$-treated cells were found in bacteria[36,77] and in mammalian cells[78,79,80] (see below for details). However, the cytotoxic action by exogenous $^1O_2$, generated by the separated-surface-sensitizer technique, was not accompanied by mutagenicity in *Salmonella typhimurium*[81]. Also, the gram-negative bacteria are more resistant to extracellular $^1O_2$ treatment than gram-positive cells[69] indicating that the outer membrane-lipopolysaccharide portion of the gram-negative cell wall makes a barrier to $^1O_2$ diffusion into the cells. These data suggest that extracellularly generated $^1O_2$ cannot reach the cellular chromosome in prokaryotic cells, what is also probably true for eukaryotic cells. Thus, the target for $^1O_2$

responsible for cell death is not necessarily DNA, at least in these cases. In human lymphocytes, however, extracellular $^1O_2$ were shown to induce low, but significant levels of sister chromatid exchanges[73]. The explanation for these results may be that $^1O_2$ damages cell membranes leading to formation of secondary lipid free radicals, which in turn might react with DNA[82].

The target which reacts with $^1O_2$ causing cell death is still a controversial matter. If the $^1O_2$ source is based on molecules that enter the cells and interact directly with nucleic acids, such as several photosensitizers, DNA damages may be responsible for at least part of cytotoxicity by $^1O_2$. Alternatively, the reactivity of $^1O_2$ with membrane causing lipid peroxidation[83], the reduction of fatty acid utilization[84] and mitochondrial damage[85,86] by photosensitization may play a significant role on the cytotoxicity of $^1O_2$.

MUTAGENICITY

The lack of mutagenesis in 26 *Salmonella typhimurium* strains exposed to $^1O_2$, generated outside bacteria, was reported by Dahl *et al.*[81]. However, working with the same bacteria species, Epe *et al.*[77] have strong evidence that $^1O_2$ is the ultimate reactive species in the pronounced mutagenicity induced by methylene blue and light. These contradictory results may be easily explained by the fact that, in the first work, the cell wall obstructs the $^1O_2$ diffusion inside the bacteria. If $^1O_2$ is produced close to the bacterial chromosome it may induce premutagenic lesions. Experiments with rodent cells treated with photosensitizers give more conflicting results. A pronounced increase in the frequency of ouabain resistant mutants (dominant marker at the Na+/K+ ATPase locus) was obtained in Chinese hamster lung cells treated with photosensitized rose bengal immobilized on polystyrene beads[78]. This mutagenic effect was enhanced by D_2O and reduced by specific $^1O_2$ quenchers, suggesting the involvement of this active oxygen species. Similar observations were made using mouse lymphoblasts exposed to photosensitized chloroaluminum phthalocyanine[79] and Photofrin II[80]. The mutagenicity was determined at the heterozygous thymidine kinase (*tk*) locus by selection of trifluorothymidine resistant cell colonies. Nevertheless, photosensitization with chloroaluminum phthalocyanine[71], hematoporphyrin derivatives[87] and Photofrin II[88] were found to be only weakly mutagenic (not significant when compared to controls) in Chinese hamster cells selected for mutations at the Na+/K+ ATPase (ouabain resistance) and hemizygous hypoxanthine phosphoribosyltransferase (*hprt*, 6-thioguanine resistance) target genes. The reason for such discrepancies is still not clear. It may be related to differences between cell strains, target genes or photosensitizers employed in each work. In any case, the ultimate interaction of $^1O_2$ with genetic material in these experiments is difficult to prove, so that interpretation of such data does not clarify the role of this excited molecule as mutagen in mammalian cells.

Exogenous DNA probes, which are exposed to $^1O_2$ outside the cell, have recently being employed in order to obtain information on the mutagenicity of $^1O_2$-induced DNA lesions. The replicative form (double stranded) DNA of the M13mp19 bacteriophage was found to be mutated at the *lacZ* target gene by pure $^1O_2$, generated by the separated-surface-sensitizer method, after replication in *E.coli*[59]. This is a forward mutational assay that can detect all classes of mutagenic events in this non essential target gene. The M13mp19 phage carries a portion of the *lacZ* gene, that produces an active β-galactosidase by α-complementation in a special bacterial host. As a result, when plated in the chromogenic substrate X-Gal, M13 clones are bright blue. Mutations in the *lacZ*α sequence in the M13 DNA may cause loss of the β-galactosidase activity and yield white and light blue M13 clones. The mutants are then screened and the *lacZ*α locus can be further investigated by DNA sequencing. Decuyper-Debergh *et al.*[59] have exploited this elegant system to obtain the mutation spectrum induced by $^1O_2$ in this DNA sequence. They found that mutagenicity by $^1O_2$ was largely due to single and double base substitutions. The most frequent base substitution (63%) was the G:C to T:A transversion, and 64 out of 65 mutants involved G:C base pairs at the parental DNA. More recently, McBride *et al.*[89] have employed the *lacZ*α mutational assay to analyse the mutagenicity of methylene blue plus light in single stranded M13mp2 DNA. Contrary to what was observed in double stranded DNA substrate[59], mutagenicity was highly dependent on the SOS response of *E.coli*. But most of the mutations was also single-base substitution involving guanine (90%). The predominant kind of substitution was G to C transversion (60%), but G to T was also found to be very frequent (25%). The main advantage of single stranded DNA plasmids is that there is no ambiguity on which strand is damaged, so that the mutation can be directly associated to the base present in the original target gene. These data suggest that mutagenicity in *E.coli* mediated by $^1O_2$-induced damaged DNA is targeted to modified guanine residues.

In mammalian cells, SV40-based shuttle vectors have been employed to investigate the mutagenicity of $^1O_2$-induced DNA lesions. The system has already been described in detail[61]. In usual experiments, the vector, either in the single or double stranded DNA structure, is treated *in vitro* with $^1O_2$, generated by the thermodissociation of $NDPO_2$, and then introduced into monkey COS7 cells, where it is replicated extrachromosomally. After a few days, the extrachromosomal DNA is isolated from the cells and shuttled back into *E.coli*. The well known genetic of this bacteria can then be used for the screening of plasmid mutations. The vectors carry the tRNA suppressor *supF* gene, whose product suppresses an amber mutation in the chromosomal *lacZ* gene, present in the special host used. As a result, the bacteria express an active β-galactosidase that can metabolize the indicator dye XGal, present in the medium, yielding bright blue colonies. Mutations in this target gene that inactivate the *supF* tRNA lead to the formation of white or light blue colonies, which can be easily distinguished among the non mutated bright blue colonies. Experiments with $^1O_2$-treated shuttle vectors have demonstrated that the induced DNA damage is highly mutagenic in mammalian cells. This mutagenicity was observed for both single[48] and double[47] stranded shuttle vectors. As for the M13 phage described above, the plasmids containing mutations were isolated and the DNA sequencing of the *supF* target gene provides the mutation spectrum induced by $^1O_2$-treatment[90]. This work revealed that most of the mutations were single and multiple base substitutions and that most of these point mutations involve G:C base pairs (98%), including G:C to T:A (51%) and G:C to C:G (33%) transversions. The same approach employing $^1O_2$-damaged single stranded DNA vectors indicated that single and multiple base substitutions are predominantly induced by $^1O_2$ (Ribeiro et al., in preparation). Most of the base substitutions found were transversions involving G (G to T followed by G to C). These data clearly suggest that, as for bacteria, the $^1O_2$-damaged guanine residues can be processed in mammalian cells by an error prone system that generates mutations.

The data obtained for both the M13*lacZ*/*E. coli* and the mammalian shuttle vector systems suggest that G to T transversions are an important kind of mutation induced in $^1O_2$-damaged DNA. Several evidences indicated that 8-oxodG, the main base modification induced by $^1O_2$ in DNA (see above), may be responsible for this kind of mutation. Shibutani *et al.*[57] found that DNA polymerases from *E.coli* (Klenow fragment) and mammalian cells (DNA polymerases α, β and δ) misincorporate dAMP opposite 8-oxodG in the template, dur-

ing *in vitro* DNA synthesis. Experiments with M13 phage[91] or plasmids[92] carrying an unique 8-oxodG residue, transfected in *E.coli*, resulted in G to T transversion mutation at the original position of 8-oxodG. The miscoding properties of 8-oxodG were also investigated by Cheng *et al.*[93] using two different M13 bacteriophage systems. Their results show that this guanine derivative has the mutagenic ability to base pair with adenine in *E.coli*. Moreover, NMR structural studies show that 8-oxodG, in the *syn* position, may base pair with dA(*anti*) in a stable DNA duplex[94] (figure 5). The existent data provide a strong support to the idea that 8-oxodG is an important premutagenic lesion in $^1O_2$-damaged DNA processed in both pro and eukaryotic cells. This mutagenicity may result as a direct error prone bypass of such lesions, by mispairing of 8-oxodG and deoxyadenosine.

A second important kind of mutation observed in $^1O_2$-damaged vectors replicated in both bacteria and mammalian cells is the G to C transversions. In at least one report[89], this base substitution is the most frequent mutation. Due to the specific mispairing of 8-oxodG with deoxyadenine, it seems highly unprobable that this lesion would also be responsible for this kind of mutation. Other lesions induced by $^1O_2$ on DNA, such as DNA cleavage, alkali and piperidine-labile sites, 4-hydroxy-8-oxodG, cyanuric acid and FapyG, may favor the G:G mispair causing G to C transversions. Although these lesions are induced less frequently than 8-oxodG, their role in $^1O_2$-mutagenicity is not known and must be considered. Alternatively, specific error prone DNA repair systems may be activated by the introduction of $^1O_2$-damaged DNA within the cell and cause preferentially this kind of mutation by dG mispairing. This is consistent with the findings of McBride *et al.*[89], who observed G to C transversions only in bacteria with the SOS processes activated by previous UV-irradiation.

CELL TOLERANCE AND DNA REPAIR OF DAMAGES INDUCED BY SINGLET OXYGEN

Several lines of evidence point to the existence of efficient DNA repair processes associated with the excision of $^1O_2$-induced DNA damage. In bacteria, it was found that DNA modifications induced by $^1O_2$ are recognized by DNA repair endonucleases present on crude extracts of *Salmonella typhimurium* and *Micrococcus luteus*[95]. This was detected by gel electrophoresis analysis of the conversion of supercoiled DNA to the relaxed open circle form after extract incubation of PM2 phage DNA treated with three different sources of $^1O_2$ (photosensitized methylene blue and rose bengal and $NDPO_2$). Employing the same methodological approach, Müller *et al.*[96] have also found endonuclease activity on DNA exposed to methylene blue and light or $NDPO_2$ in *E.coli* extracts. Cell extract from bacterial strain defective in the formamidopyrimidine-DNA glycosylase (FPG) do not cleave $^1O_2$-damaged DNA. Moreover, experiments with purified FPG protein confirm that this enzyme recognize and cleave DNA exposed to $^1O_2$. These data demonstrate the participation of FPG enzyme on the repair of DNA bases modified by $^1O_2$. More recently, Boiteux *et al.*[53] have investigated the excision of the modified bases from $^1O_2$-damaged DNA by FPG protein. The authors found that FPG protein excised almost exclusively 8-oxodG and FapyG, two important lesions induced by $^1O_2$. The role of this protein on the *in vivo* DNA repair of $^1O_2$-induced lesions was further supported by experiments in which plasmid DNA was exposed to methylene blue and light and then transfected into different *E.coli* strains. Plasmid inactivation was significantly increased in the double mutant *E.coli* strain deficient in the *fpg* and *uvrA* genes, when compared to wild type and *fpg* or *uvrA* single mutants[97]. Furthermore, *in vitro* cleavage of damaged plasmid DNA by the purified FPG and UVRABC repair enzymes was also observed by these authors. Therefore, *E.coli* DNA excision repair pathways mediated by FPG and UVRABC enzymes contribute to the repair of lethal damage induced by $^1O_2$. Another endonuclease activity of *E.coli* was reported to remove specifically 8-oxodG[98]. However, further analysis of this, so called, 8-oxodG DNA glycosylase suggested that it is identical to the FPG protein[99]. Recent data have implicated at least two other bacterial genes in the processing of 8-oxodG. Deficiency in these genes result in mutator strains in *E.coli*, so they are called *mutT* and *mutY*. The MutT protein acts on the nucleotide pool for DNA synthesis degrading 8-oxodGTP to monophosphate. In the absence of this protein the oxidized guanine can be incorporated in DNA causing the mutator phenotype by yielding A:T to C:G transversion[100]. On the other hand, Michaels *et al.*[101] have obtained evidence that the MutY protein removes undamaged adenines from DNA when it mispairs 8-oxodG during DNA replication. This activity is fully complemented by the FPG protein, which removes the 8-oxodG from the mispair dA:8-oxodG. As a result the single mutant *mutY* and the double mutant *mutYfpg* have elevated rates of spontaneous mutation frequency, and mutations were genetically identified as G:C to T:A transversions.

In eukaryotic cells, much less is known on the DNA repair processes involved in the metabolism of $^1O_2$-induced DNA lesions and some evidences for their existence are indirect. In yeast, the *rad18* strain exhibit a mutator phenotype which was characterized to be due to G:C to T:A transversions[102]. It seems that this strain is unable to correct G:A and C:T mismatches. It would be interesting to check how this yeast strain handles with DNA containing 8-oxodG mispairing with dA. For mammalian cells, Feldberg *et al.*[103] have described a damage-specific DNA binding protein from human cells, which recognizes a substrate on guanine-containing nucleic acid polymers exposed to methylene blue and visible light in D_2O buffer. The amount of these protein binding sites decrease if the polymers are treated in the presence of H_2O or azide, suggesting that guanine oxidation proceeds at least in part by a $^1O_2$ mechanism. An enzyme with glycosylase activity similar to the FPG protein has been isolated from mammalian

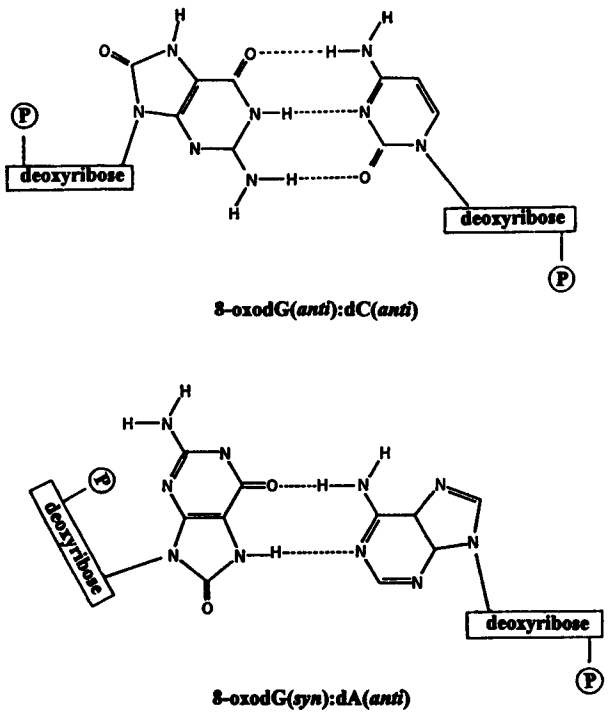

Figure 5. Possible base pairing between 8-oxodG and dC or dA.

cells[104]. Also, an endonuclease activity that removes specifically 8-oxodG from DNA has also been reported in human polymorphonuclear neutrophils[105]. The relationship between these findings remains to be established. However, the elimination of 8-oxodG from damaged DNA in mammalian cells is almost certain. Kasai *et al.*[106] have found that the amount of this lesion decreases with time after γ-irradiation, suggesting the excision repair of 8-oxodG. The removal of 8-oxodG by excision from cell DNA *in vivo* is further supported by the detection of this modified guanine residue in urine[107,108,109]. Moreover, Fraga *et al.*[110] measured the 8-oxodG in DNA from different organs from rats of different ages. They found an age-dependent increase of this lesion in liver, kidney and intestine, but not in brain and testes. The excretion of 8-oxodG in the urine of these animals decreased with age. Thus, the accumulation of 8-oxodG with age may be related with a decrease of DNA repair activity or, alternatively, to an increase of oxidative DNA damage. Finally, a higher mutability per damage induced by $^1O_2$ is observed in double stranded vectors transfected into mammalian cells, as compared to single stranded DNA[62]. Since single stranded DNA is probably not repaired, due to the absence of a template, these data may be explained by the existence of an error prone DNA repair in mammalian cells which acts on double stranded DNA carrying $^1O_2$-induced lesions. This repair would be very efficient in preserving the DNA biological activity, but is highly mutagenic.

CONCLUDING REMARKS

There is a growing interest on the interaction of $^1O_2$ with DNA and its biological consequences. The ability of $^1O_2$ to damage DNA *in vitro* with its genetic effects is becoming widely accepted, and the possibility that this reaction takes place within the cell is still controversial, but it is certainly a matter of concern. The high reactivity of $^1O_2$ in biological systems raises the question if it can ever reach the genetic material. Several biological compounds have been shown to deactivate $^1O_2$, such as carotenoids[111,112], lycopene[113], tocopherols and biological thiols[114,115], imidazole compounds[116], fatty acids[117] and human plasma[118]. More recently, DNA was found to be protected from $^1O_2$ attack *in vitro* by spermine and spermidine, wellknown constituents of the eukaryotic chromatin[119]. Thus, $^1O_2$ probably has to be generated very close to DNA to cause any damage. Evidently, the elevated number of DNA repair systems described at least in *E.coli* and the fact that an important kind of spontaneous mutation *in vivo* (such as G to T transversion) is found to be a major mutagenic consequence of $^1O_2$-induced DNA damage point to a possible important deleterious role of this excited molecule *in vivo*. However, the demonstration of spontaneous $^1O_2$ activity on DNA inside the cell inducing genetic damage remains a challenge.

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