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Invited Review

REPAIR OF DNA DAMAGE INDUCED BY REACTIVE OXYGEN SPECIES

LARS H. BREIMER

Department of Chemical Pathology and Human Metabolism, Royal Free Hospital School of Medicine, Pond Street, London NW3 2QG, UK

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DNA repair limits the mutagenic, and thereby the carcinogenic, effect of DNA modifications. Free radicals, particularly reactive oxygen species, induce all forms of DNA damage, including base modifications, base free sites, strand breakage, and cross-links. These lesions are repaired by a variety of enzymes of partly overlapping substrate specificity, some of which may be induced.

KEY WORDS: DNA repair, mutagenesis, free radicals.

INTRODUCTION

Cellular DNA continually suffers premutagenic modification owing to hydrolysis, methylation, and reactive forms of oxygen.[1-3] As DNA is the template for its own synthesis, failure to repair lesions would result in unacceptable mutation rates and, ultimately, demise or in the case of mammalian cells, malignant transformation. *E. coli* has proved to be a valuable system for investigating repair processes, and has provided a conceptual framework to approach DNA repair in mammalian, including human, cells, where enzymes are generally present at much lower levels.

The chemistry of, and mutagenic and carcinogenic effects of free radicals have been the subject of several reviews.[2-14] Here I review the processes involved in the repair of DNA damaged by reactive oxygen species. It is useful to distinguish between *repair* of (otherwise) permanent lesions, *restitution* of intermediates to the pre-injury state, and *protection* against damage. The detailed description of processes involved in restitution of transient intermediates as well as those preventing damage (e.g. by quenching radicals) are beyond the scope of this review, as are more general processes preserving the integrity of the DNA sequence such as mismatch repair and proofreading by DNA polymerase. Previous reviews have concentrated on the repair of radiation induced damage.[12-14]

Excision repair is the main pathway of repair of DNA damage caused by reactive oxygen species (Table I). Direct reversal of a modification is not known to be used. Recombinational processes are necessary to deal with double strand breaks and crosslinks. Finally, defects in the religation following repair synthesis may present a phenotype reminiscent of increased sensitivity to the genotoxic effects of free radicals. It is convenient to consider DNA repair in terms of the modifications induced (Table II).

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TABLE I
Simple classification of DNA repair systems described in *E. coli* relevant to free radicals

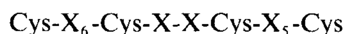
Repair type	Enzyme examples	Comments
Excision repair	DNA glycosylase AP endonuclease	Commonly used in free radical repair
Reversal repair	Photoreactivating enzyme O^6-alkylguanine-alkyltransferase	Not used in free radical repair
Recombination repair	RecA protein	Repair of strand breakage and cross-links
Oligosynthesis	DNA polymerase	Proof-reading of DNA polymerase and mismatch repair beyond scope of review
Religation	DNA ligase	Central role in DNA repair (and replication)

Г. Repair of Base Modifications

a) Ring-damaged pyrimides The excision of the ring-saturated, ring-contracted, and ring-fragmented derivatives of thymine (*cis*- and *trans*-thymine glycol, 6-hydroxy-dihydrothymine, 5-hydroxy-5-methylhydantoin, urea and methyltartronylurea) from damaged DNA are primarily functions of DNA endonuclease III in *E. coli.*[15-17] This enzyme releases the modified bases by a DNA glycosylase action (DNA glycosylases are enzymes which cut the base-sugar bond of modified bases to release the base in free form.[1] They generally prefer double-stranded DNA and have few cofactor requirements.) Endonuclease III also cuts the phosphodiester bond on the 3′ side of the resultant base-free sugar, that is it acts as a 3′ AP endonuclease (see below). It incises DNA not only at damaged thymines but also modified cytosines and guanines.[20-23] The guanine lesion(s) involved have not been identified, but could be urea because urea is a potential end product of all oxidative base damage (reviewed in [12]). It has also been reported that endonuclease III releases an unidentified cytosine UV-photoproduct, in addition to cytosine glycol.[24-26]

Endonuclease III was first described by Radman as an activity which incised heavily UV-irradiated double-stranded DNA.[27] This is due to its action on 6-hydroxydihydrothymine, a minor UV-photoproduct.[17] Endonuclease III is encloded by the *nth* gene,[28] which has an open reading frame of 633 bp that codes for 211 amino acid residues.[29] It is a monomeric globular protein of predicted M_r = 26 kDa, which is in good agreement with physical determination, and the enzyme contains 4 iron ions per molecule, arranged in an $[4Fe{-}4S]^{2+}$ cluster.[16,30]

Endonuclease III is the only DNA glycosylase to show sequence similarity with another protein: MutY.[31] MutY, the product of the *mutY* gene, is an adenine-DNA glycosylase which acts in mismatch repair to excise the adenine of a *G·A* mispair. These two proteins share in their carboxyterminal half a sequence of 181 amino acids that are 2/3 similar and 1/4 identical, including specifically the sequence


Cys-X_6-Cys-X-X-Cys-X_5-Cys

which probably accounts for the Fe–S cluster, generally encloded

Cys-X-X-Cys-X-X-Cys.

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TABLE II
Repair of lesions of base residues in DNA induced by free radicals

Lesion	*Base*	*Enzyme*	*Comment*
Pyrimidine hydrates	T & C	Endonuclease III: combined DNA glycosylase and 3′ AP endonuclease	Thymine glycol is a poor substrate for UvrABC excinuclease
5-Hydroxy-5-methyl hydantoin	T	Endonuclease III: combined DNA glycosylase and 3′ AP endonuclease	
Urea	all	Endonuclease III: combined DNA glycosylase and 3′ AP endonuclease	
Methyltartronylurea	T	Endonuclease III: combined DNA glycosylase and 3′ AP endonuclease	
Imidazole ring opened purine	A & G	*fpg* gene product: combined DNA glycosylase and 3′ & 5′ AP endonuclease	
5-Hydroxymethyluracil	T	DNA glycosylase	mammalian cells; ? true substrate
5-Hydroxymethylcytosine	meC	DNA glycosylase	mammalian cells; ? gene regulation
Base-free sites	all	AP endonucleases	poor substrate for UvrABC exincuclease
8-Hydroxypurines	A & G	not known	

The mammalian equivalent of endonuclease III was probably first described by Bacchetti and Brent and their collaborators,[32–34] (reviewed in [35]). It has been characterised from a number of sources including calf thymus,[21,36] human and rodent fibroblasts and lymphocytes,[36–39] and yeast.[40] A curious feature of this enzyme is that its preferred substrate appears to be urea[36] but the reason for this preference is not clear. It may reflect the observation that urea residues in DNA can be derived from any base. Surveys of human cells derived from patients suffering from inherited disorders characterised by increased sensitivity to ionising radiation and risk of cancer have failed to reveal any deficiency.[23,36]

E. coli mutants deficient in endonuclease III (*nth*$^-$) are not more sensitive to the lethal effects of H_2O_2 and ionising radiation, though they suffer from mildly increased spontaneous mutation frequencies.[28] Van Houten *et al.* and Sancar *et al.* have independently demonstrated that thymine glycol in DNA is excised by UvrABC excinuclease, an enzyme primarily concerned with the repair of pyrimidine dimers and bulky adducts to bases.[41,42] UvrABC is a multi-subunit enzyme which cuts the strand containing the base modification about 8 bases upstream and 4 bases downstream of the lesion which is released as part of a 12–13mer oligonucleotide.[19]

b) Imidazole ring open purines Hydroxyl radical, and possibly singlet oxygen, attack on the imidazole ring of purines results in saturation and fragmentation to give 5-formamidopyrimidine derivatives.[4–6] A formamidopyrimidine is also generated by alkaline hydrolysis of 7-methylguanine in DNA.[43] Imidazole ring opened purines are excised by formamidopyrimidine-DNA glycosylase, which is encoded by the *fpg* gene.[43–45] There is a mammalian equivalent.[36,46]

The *fpg* gene (807 bp, 269 amino acid residues) encodes a protein of predicted M_r = 30 kDa. This enzyme also acts as an AP endonuclease, but catalyses a combined *beta-delta* elimination reaction both 5′ and 3′ to the AP site.[45,47–49] Mutants deficient in *fpg* are not more sensitive to the lethal effects of ionising radiation or H_2O_2 indicating that other pathways must be able to cope with these lesions.[50]

c) 8-Hydroxyguanine 8-Hydroxyguanine in DNA is now strongly implicated in the action of certain carcinogens (for review see refs[3,11,12]). It is the only base modification induced by reactive oxygen species which has been shown to miscode directly not only opposite itself but also at neighbouring bases.[51] Thus 8-hydroxyguanine can potentially cause paired amino acid substitutions, and would consequently not necessarily be detected in reverse mutagenesis tests. (It is possible that 8-hydroxyadenine may act likewise.) This is in contrast with the ring-damaged pyrimidines and purines which generally block DNA polymerase.

8-Hydroxyguanine can probably be generated by the action of 1 singlet oxygen, although if the flux of singlet oxygen is excessive strandbreakage can occur because of a second hit on the modified guanine.[52,53] Exposing DNA to visible light in the presence of methylene blue dye appears to generate not only 8-hydroxyguanine in DNA but also other lesions, including FaPy Gua.[53] Two studies in prokaryotes have been reported that show that singlet oxygen can cause mutations[54,55] although the lesions in DNA were not identified and the treatment was comparatively toxic, probably because of cell membrane damage. These studies need to be complemented with studies in mammalian systems such as the Chinese hamster ovary (CHO) cell line *aprt* system developed by Meuth (reviewed in refs[3,12]), though toxicity is likely to be

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an even greater problem. Intranuclear "caged" release of single oxygen would be an advantage.[56]

The repair of 8-hydroxyguanine in DNA is not yet understood, mainly because no suitably radioactively labelled guanine derivatives are available commercially, only 8-^{3}H guanine. As an alternative, the possibility of using electrochemical determination to monitor the disappearance of the lesion from model substrate should also be explored.

Indirect evidence[55] suggests that a base lesion induced by visible light/methylene blue is excised by a DNA glycosylase, but that it is also a poor substrate for UvrABC exinuclease. This lesion is likely to be imidazole ring opened purine as it is a substrate for the FPG protein but not other DNA glycosylases (53 and Boiteux and Laval, personal communication).

5-Hydroxymethylurcil-DNA Glycosylase

If 8-hydroxyguanine is a pernicious base modification waiting for its repair enzyme to be identified, then 5-hydroxymethyluracil-DNA glycosylase would appear to be an enzyme in search of its true substrate. A DNA glycosylase activity for 5-hydroxymethyluracil was first reported in a rodent lymphoblastoid cell line.[38] Teebor's group, in addition to characterising 5-hydroxymethyluracil-DNA glycosylase, has also identified an activity acting on 5-hydroxymethylcytosine.[57–59] Both activities appear only to be present in terminally differentiated mammalian cells and are the only DNA glycosylases not identified in prokaryotes.

Teebor and co-workers have purified the 5-hydroxymethyluracil-DNA glycosylase 1800-fold from calf thymus.[59] It has M_r = 38 kDa, and is equally active on a single-stranded as on double-stranded DNA. This is unusual since glycosylases tend to prefer one form, usually double stranded. They also claim that 5-hydroxymethylcytosine-DNA glycosylase is a distinct enzyme, although no data are presented.

5-Hydroxymethyluracil is the natural constituent of certain bacteriophages where it replaces and codes for thymine.[60] This could explain why it is not detected in bacteria. Feeding cells in culture 5-hydroxymethyldeoxyuridine has been reported to be toxic and cause mutations.[59] Apparently it is incorporated into the DNA. However, medium supplementation with nucleoside is notorious for causing nucleotide pool imbalances which are mutagenic.[61]

One possible rationalisation of the data about this enzyme is that 5-hydroxymethyluracil is not its true substrate, but that it recognizes some other form of modification of the 5-methyl group. That modification may interfere with DNA transcription, either through affecting RNA polymerase action, or by interfering with transcription factor binding to sequences in the promoters or enhancers. The need to remove 5-hydroxymethylcytosine would be related primarily to the regulation of gene activity. Candidate lesions would be amino-acid crosslinks or 5-hydroxyperoxymethyl derivatives.[62,63]

2. Repair of Base Free Sites

Reactive oxygen species induce base free, also known as apurinic/apyrimidinic (AP), sites, which are mutagenic (for review see [64–68]). They also result from the action of DNA glycosylases, although those acting on reactive oxygen induced damage probably never generate free AP sites.[1,20,49]

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The AP endonucleases of *E. coli* fall essentially into two groups[69]: those which cut the phosphodiester bond on the 5′-side of the AP site, and those which cut on the 3′-side and which have associated DNA endonuclease activity. Both enzymes generate 3′-hydroxyl ends, but 3′-hydroxyl base free sugars are poor primers for DNA polymerase, and 5′-base free sugars must be removed (see below).

The main AP endonuclease activity of *E. coli* is the multifunctional enzyme exonuclease III, which accounts for more than 90% of the cells AP endonuclease activity.[18,19] It is a 28 kDa protein, requires Mg^{2+} for activity, and is encoded by the *xthA* gene. Exonuclease III has four functions: 1) 5′ AP endonuclease; 2) 3′ to 5′ exonuclease; 3) excision of 3′-phosphate, 3′-terminal deoxyribose 5′-phosphate, and 3′-phosphoglycolate esters; 4) RNaseH action, which acts in DNA replication.

Endonuclease IV is an EDTA resistant 5′ AP endonuclease originally characterised by Ljungquist in exonuclease III deficient (*xthA*⁻) mutants[70]. It is encoded by the *nfo* gene[68] and also acts in 3′ end preparation (see below).

The 3′ AP endonuclease activity appears only to be associated with DNA glycosylase activity (see above). It is a *beta*-elimination reaction rather than a true phosphodiesterase activity. The role, if any, of endonucleases V and VII is reactive oxygen species repair is not clear. Laval and coworkers have estimated that of the EDTA resistant AP endonuclease activity in *xthA*⁻ mutants, 60% is due to *nth*, 10% to *nfo* and 20% to *fpg*.[47] (UvrABC exinuclease can act on DNA containing base free sites but requires Mg^{2+} and ATP [19].)

Mammalian AP endonucleases are generally similar to exonuclease III. They are about 28–32 kDa in size and require Mg^{2+} for activity, but are monofunctional.

3. *Preparation of Modified Ends for Repair Synthesis*

Hydroxyl radical action on (eg *gamma*-irradiation of) DNA *in vitro* produces two types of single strand breaks.[71–74]. Both contain 5′-phosphate termini. The 3′ termini can be either phosphates or 3′-phosphoglycolates, neither of which act as primers of *E. coli* DNA polymerase.

a) 3′-ends Elegant work by Demple's group has demonstrated that exonuclease III and endonuclease IV (and their eukaryotic homologue) *in vivo* trim modified 3′-ends so that these can act as primers for DNA polymerase.[75–84] Briefly, Demple noticed that *E. coli xthA*⁻ mutants, which are deficient in exonuclease III, were highly sensitive to the lethal effects of H_2O_2 and *gamma*-rays.[75] It was known from work in Haseltine's laboratory that *E. coli* exonuclease III *in vitro* could hydrolyse DNA with *gamma*-ray-induced 3′-end modifications.[74] Demple went on to show that *xthA*⁻ mutants accumulated single-strand breaks in DNA that, when isolated from the bacteria, were poor primers for DNA polymerase *in vitro* but could be converted into good primers by incubation with pure exonuclease III.[76] Model substrates containing 3′-terminal deoxyribose 5-[^{32}P]phosphate and 3′-phosphoglycoaldehyde were used to show that exonuclease III could remove whole nucleotides, 3′-phosphates, deoxyribose-5-phosphates, and the phosphoglycolate esters formed by *gamma*-rays *in vitro*. In *xthA*⁻ mutants about half of the activity needed to prepare 3′ ends for repair synthesis resided in endonuclease IV, the remainder being the function of poorly characterised enzymes of ca 40–55 kDa (which may be identical to deoxyribosephosphatase – see below), and 25 kDA (possibly endonuclease V) and 28 kDa respectively.[76–78]

Endonuclease IV can be induced 10-fold by paraquat acting through superoxide

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TABLE III
Sensitivity of *E. coli* mutants to DNA damaging agents

Genotype	viable	Sensitivity to				intrachromosomal recombination
		H_2O_2	ionising radiation	bleomycin	MMS	
xth⁻	yes	+ + +	+	−	+	↑15 ×
nfo⁻	yes	−	+	+ +	+	↑ 2 ×
xth⁻ *nfo*⁻	yes	> xth	+ +	= nfo	+ +	↑50 ×
uvrA⁻ *xth*⁻ *nfo*⁻	no					
recA⁻ *xth*⁻ *nfo*⁻	no					
xth⁻ *dut*⁻	no					
xth⁻ *dut*⁻ *nfo*⁺ × *10*	yes					
xth⁻ *dut*⁻ *ung*⁻	yes					↑

Genes: xth = exonuclease III; nfo = endonuclease IV; uvrA = DNA binding part of ABC exonuclease; recA = recombination A protein; dut = dUTPase; ung = uracil-DNA glycosylase

NB In vivo endonuclease IV acts on lesions that are poor substrates for exonuclease III. These may be oxidized AP sites.

radical.[79] It is part of the *soxR* regulon, which is mediated by the superoxide ion, and which also includes Mn-SOD and glucose-6-phosphate dehydrogenase.[80]

Yeast contains an enzyme encoded by the *Apn1* gene that is similar to endonuclease IV though not inducible.[81-83] Whereas there are about 50 molecules of endonuclease IV in an uninduced cell, rising to more than 500 on induction, yeast contains about 7,000 molecules of APN1 enzyme per cell. It has M_r = 41 kDa. This enzyme appears to perform both the functions that reside in exonuclease III and endonuclease IV in *E. coli*. When the *Apn1* gene is expressed in *E. coli* it restores *xthA*⁻ *nfo*⁻ double mutants to wild type phenotype.[84] This demonstrates that the enzyme can act over a great phylogenetic distance. The availability of the gene of this lower eukaryote enzyme should facilitate the cloning of the human gene.

b) 5′-ends The majority of AP endonuclease action results in 5′-phosphate attached to the base free sugar. These must be excised. In *E. coli* this is carried out by, 2-deoxyribose-5-phosphatase, dRPase.[85] It is a 55 kDa protein which requires Mg^{2+} and acts optimally at pH 6.5. It may be identical to the larger of the enzymes described by Bernelot-Moens and Demple.[86]

c) Is the carcinogenic process of alcoholic liver disease an effect of overwhelming of the 3′ end repair system? The carcinogenic process of alcoholic liver disease has been proposed to be mediated by free radicals, probably involving acetaldehyde and, possibly, formaldehyde.[87-90] Lin *et al.*[89] have shown that ethanol and acetaldehyde potentiate the clastogenic effects of UV light, methyl methanesulphonate, mitomycin C and bleomycin in CHO cells in culture. This is reminiscent of the behaviour of various combinations of *E. coli xth*⁻ and *nfo*⁻ mutants (cf Table III). Thus it is possible that these events occur through an overload of the 3′ end preparation pathway, either by generating more damaged 3′ ends (eg through increased base damage, AP sites, or direct action) or by interfering with their repair. In either case the half-life of single-strand breaks would be longer, leading to an increased risk of a nearby lesion which could increase the frequency of double-strand breaks. The persistence of double-strand breaks owing to failure of repair correlate with increased

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frequency of chromosome aberrations.[91,92] However, the mechanism of mutagenesis of known free radical agents such as *gamma*-rays appears to be locus and system specific and point mutations account for a considerable proportion (reviewed in [3,12]).

4. Recombination Repair

The process generally referred to as recombination repair is not one of true repair but rather a means of tolerating a DNA lesion until it can be repaired.[69] It is also known as "daughter strand gap repair" and "post-replication repair". It involves the action of recA protein. Recombination processes are necessary for repair of double-strand breaks, as well as crosslinks (which also require UvrABC action) (see above).

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5. Bloom's Syndrome

Bloom's syndrome is one of a number of rare inherited human disorders associated with an increased risk of cancer. Such syndromes can be regarded as naturally occurring (viable) human mutants. Just over 100 cases of Bloom's syndrome are known world-wide and about 30% of them have developed cancer.[93] Its inheritance is autosomal recessive, and the patients belong to one complementation group[94] which argues that a defect in a single locus is the cause.

Cells obtained from sufferers of Bloom's syndrome are characterised by an elevated frequency of chromosomal aberrations and spontaneous sister chromatid exchanges.[95-97] When cells are cultured in D_2O which prolongs the half-life of some reactive oxygen species, particularly singlet oxygen, those from Bloom's syndrome show a small elevation but those from Fanconi's anaemia patients show a marked elevation of chromosome breakage.[98,99] When Bloom's cells are tested in culture they show 10-fold elevated spontaneous mutation frequencies.[100] A free radical mechanism has been proposed to explain the pathology of Bloom's syndrome.[101,102]

Gianelli and co-workers observed that replicative intermediates of DNA took longer in Bloom's cells than in normal cells to mature into high molecular weight forms[103] and proposed that in Bloom's syndrome there was a defect in DNA replication. Indeed, Bloom's cells show decreased rate of DNA replication fork displacement.[104] Experiments with DNA damaging agents suggested that Bloom's cells suffer from abnormality of the post-incision step of DNA-excision repair.[105]

An explanation alternative to that of free radical damage has been provided by Willis and Lindahl who described a deficiency in the activity of DNA ligase I.[106,107] This has been independently confirmed.[108]

DNA ligase I has now been purified to homogeneity from calf thymus, and partially from human cells.[109,110] It is synthesized and exists as a 125 kDa protein which can be partially degraded by proteolysis to a 85 kDa form, which is still active. A specific polyvalent antiserum has been raised, which cross reacts with mammalian, yeast and vaccinia virus DNA ligase. This will enable cloning of the human gene for DNA ligase I, which in turn will allow definitive assessment of the Bloom's defect. Until such studies are completed, however, it remains a possibility that the Bloom's defect resides in another factor closely involved in the ligation process, tightly associated with DNA ligase I, or in the synthesis of such a factor. In this context it is worth noting that Poot's laboratory has reported that non-transformed fibroblasts from Bloom's patients exhibit cell cycle kinetic disturbances different from those of permanent Epstein-Barr virus transformed B-cell lines from Bloom's sufferers.[102]

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CONCLUSION

The repair of reactive oxygen species induced DNA damage involves a wide variety of enzymes, some of which have overlapping functions. Many of the enzymes are multifunctional and have broad substrate ranges. Others can be induced. This reflects the ubiquity of and danger from this form of damage. The existence of repair enzymes for specific base damage indicates the importance of such lesions. However, the lethal effects of reactive oxygen species would, at least in bacteria and yeast, appear to be mediated mainly by single-strand breakage. Carcinogenesis involves a series of events,[111,112] which may include cell death and regeneration, and a combination of DNA modification and growth stimulation is probably necessary to achieve transformation of a cell into malignant growth.

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