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A NEW GUANINE-SPECIFIC REACTION FOR CHEMICAL DNA SEQUENCING USING m-CHLOROPEROXYBENZOIC ACID

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When radio-labelled M13mp18 DNA immobilized on a solid support was treated with 0.1M m-chloroperoxybenzoic acid at pH 6.0 followed by piperidine, chain cleavage occurred at guanine residues, suitable for sequencing.

Since the introduction of the chemical cleavage method of DNA sequencing by Maxam and Gilbert (1) there has been a continual search for new and reliable DNA modification reactions. Very many reactions are now known which lead to cleavage of DNA at one or more bases (2,3). Despite this none of the known thymine-specific reactions with permanganate, hydrogen peroxide, borohydride or photo-activators have been found to be sufficiently reliable due to their sensitivity to secondary structure. Furthermore, routine sequencing requires handling with hazardous chemicals like dimethylsulfate and hydrazine which are very toxic and potentially carcinogenic.

In an attempt to find new modification reactions we examined several organic peracids. This class of compounds are known to attack all four bases in DNA (4) but have never been used for DNA sequencing. Subharaman *et. al.* (5) made a detailed study of the action of m-chloroperoxybenzoic acid on a variety of nucleosides. All were oxidised but each showed a different pH dependence. We have reinvestigated this reagent in the sequencing context.

MATERIALS AND METHODS

m-Chloroperoxybenzoic acid (MCPBA) was purchased from Aldrich and was of 80-90% purity. Dimethylsulfate (>99%), triethylamine (>99,5%)

0006-291X/90 $1.50

and piperidine (>99%) were obtained from Fluka. Hydroxylammonium chloride (99%) was from BDH. Hybond M & G paper and [γ-^{32}P]ATP were obtained from Amersham International. Polynucleotide kinase and Sequenase 2.0 were from BRL and USB, respectively.
For G-modification we used a 0.1 M solution of MCPBA in 50% aqueous ethanol. We prepared 50 ml as stock solution. For the calculation we assumed that the compound is of 100% purity. Dissolve 8.6 g of MCPBA in 40 ml of 50% aqueous ethanol, adjust pH to 6.0 with 1 M NaOH and make up to 50 ml with 50% aqueous ethanol. The pH of the resulting solution should be checked before use and, if necessary, readjusted.

DNA labelling
M13 (-21) universal primer (10pmol) was labelled at its 5'-end with polynucleotide kinase (10 units) and [γ-^{32}P]ATP (40 µCi) according to standard procedures. 2 pmol of labelled primer were annealed to 1 µg of M13mp18 DNA in 15 µl containing 5 µl of 5 x Sequenase 2.0 reaction buffer for 5 min at 55°C and then slowly cooled to 37°C over 30 minutes. 1 µl of 100 mM DTT, 8 µl of water and 1 µl of diluted Sequenase 2.0 (5 units) were added and the mixture incubated for 5 min at 37°C.
Double-stranded plasmid DNA (2 µg) was first denatured with 0.2 M NaOH followed by ethanol precipitation. 4 pmol of labelled primer were annealed to the denatured DNA in 15 µl containing 5 µl of 5 x Sequenase 2.0 reaction buffer. Extension was carried out as described above.

Chemical DNA sequencing
The labelled DNA was incubated for 3 min at 95°C and immediately chilled on ice. 2 µl of labelled DNA were immobilized onto 4 individual 3 mm x 3 mm squares of Hybond M & G paper. The G reaction with 1% dimethylsulfate in 50 mM ammonium formate at pH 3.5, the A+G reaction with 66% formic acid, and the C reaction with 4 M hydroxylamine at pH 6.0 were performed essentially as described by Rosenthal et al (7). The G-reaction with 0.1 M MCPBA in 50% aqueous ethanol at pH 6.0 was performed with 500 µl of the reagent on the solid support for 2 minutes at room temperature. The modification reaction was terminated by washing the solid support 3 times with 25 ml of 50% aqueous ethanol in a beaker.
All modification reactions were followed by chain cleavage with 10% piperidine at 90°C.

RESULTS AND DISCUSSION

The reaction of single and double stranded DNA was investigated over a range of pH values and MCPBA concentrations. Above pH 6.0 there was little base specificity and at concentrations below 0.1M little reaction was evident (results not shown). However at 0.1M and pH 6.0 the reagent was able to modify guanine residues with almost the same specificity as does dimethylsulfate in the traditional sequencing reaction. This is illustrated in Figure 1 showing part of the degradation pattern of radio-labelled

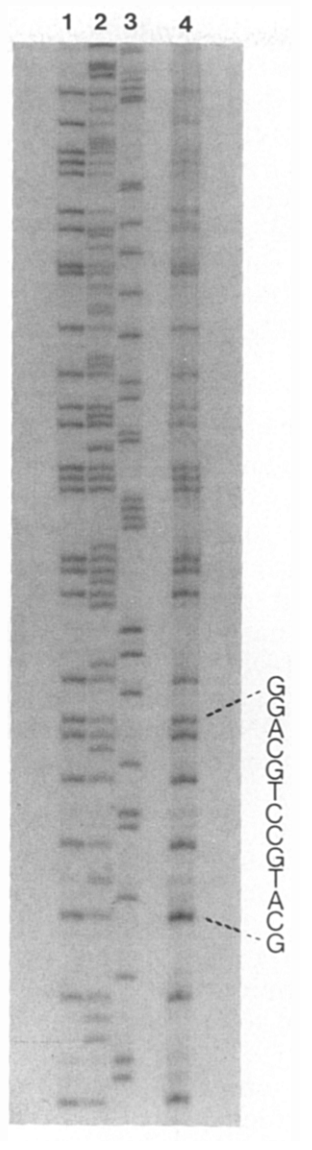

Figure 1.
Fraction of the sequencing ladder of radio-labelled M13mp18 DNA obtained by chemical degradation on solid support. Lane 1: G reaction with 1% dimethylsulfate in 50 mM ammonium formate pH 3.5; lane 2: A+G reaction with 66% formic acid; lane 3: C reaction with 4 M hydroxylamine at pH 6.0, and lane 4: G reaction with 0.1 M m-chloroperoxybenzoic acid (MCPBA) at pH 6.0.

M13 mp 18 DNA. By close inspection of various sequence ladders we have found additional faint bands at some C and A residues, in a number of cases, leading to a slightly higher overall background; this does not complicate the interpretation of the ladders. The modification specificity of the new reagent is therefore G>>A,C.

The mechanism of the oxidation is not clear. Previous studies at the nucleoside level have shown that under weakly acidic conditions A and C are oxidised by peracids to their N-oxides. The formation of these and their subsequent base-catalysed degradation may account for the feint A and C bands in the ladders. We are of the opinion that the observed G-reaction must depend on an oxidation of the 4,5- double bond, perhaps via an epoxide, with subsequent ring cleavage. It is not obvious, however, why the electrophilic OH^+ (or its mechanistic equivalent) should not also lead to degradation of thymine residues as well, as it does with the nucleoside (5).

Other oxidative G-specific reactions have been described. The methylene blue photoreaction in which $^1O_2$ is generated *inter alia* is very effective (6), but is technically less useful in the context of immobilised DNA (7). The evidence suggests that in this photo-oxidation more than one mechanism operates but that ring degradation possibly via 8-hydroxyguanine again leads to a base labile residue (8,9). Since completion of the present work the very labile and reactive dimethyldioxirane has been shown to oxidise a range of nucleosides, but, applied to DNA sequencing, it leads to preferential reaction at guanine residues (10). In this sense it resembles MCPBA. The per-acid may have the advantage that it can be purchased and is less hazardous than dimethylsulphate, the G-specific reagent normally used in the Maxam-Gilbert procedure.

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