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Pyrimidine-specific chemical reactions useful for DNA sequencing

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ABSTRACT

Potassium permanganate reacts selectively with thymidine residues in DNA (1) while hydroxylamine hydrochloride at pH 6 specifically attacks cytosine (2). We have adopted these reactions for use with the chemical sequencing method developed by Maxam and Gilbert (3).

INTRODUCTION

Maxam and Gilbert's chemical method of sequencing DNA relies on base-specific DNA modification followed by piperidine-induced β-elimination which cleaves the DNA sugar-phosphate backbone at the modified sites (3). In our experience and that of other laboratories (4) the pyrimidine-specific reactions using hydrazine described by Maxam and Gilbert (3) are, in general, less reliable than those which modify purines. NaCl addition will not suppress thymidine (T) cleavage if hydrazine which has been oxidized by repeated exposure to air is used (3). Large salt pellets occasionally persist through the two ethanol precipitations in Maxam and Gilbert's cytosine (C)-only reaction. T bands in the Maxam-Gilbert C + T reaction are often weakened by small amounts of salt carried in the DNA sample (3). We have perfected a T-specific reaction using potassium permanganate ($KMnO_4$) and a C-specific reaction using hydroxylamine hydrochloride ($NH_2OH \cdot HCl$).

These reagents, unlike the anhydrous hydrazine used in Maxam and Gilbert's procedure (3) and the osmium tetroxide used by Friedmann and Brown to modify T (4), are cheap, stable, readily available solids of low toxicity. These properties make them attractive alternative reagents for chemical DNA sequencing. We also describe a very slight modification of the Maxam-Gilbert C-only reaction using hydrazine acetate ($N_2H_4 \cdot CH_3COOH$).

MATERIALS AND METHODS

CAUTION: Potassium permanganate is a potent oxidizing agent, while hydroxylamine and hydrazine are strong reducing agents. Take care that large quantities of these chemicals do not come in contact with each other or a violent reaction may occur.

T-Specific Modification by $KMnO_4$

The $KMnO_4$ (Mallinckrodt) reagent was prepared as a 1.27×10^{-2} M 100x stock solution in twice-distilled water. When stored under refrigeration and in darkness, the stock solution is stable for at least one week. The 100x stock solution is diluted to 1.27×10^{-4} M as needed.

A 5 µl aliquot of labelled DNA (roughly 10-15% of the total cpm in the sample) in water and containing 2-5 µg of unlabelled carrier DNA was denatured by heating at 90°C for 2 minutes followed by quick-chilling in ice water. Twenty microliters of 1.27×10^{-4} M $KMnO_4$ were added and the mixture was incubated at 20°C. The mixture will appear pink. The fragment shown in Figure 1 was 300 nt long and the incubation period was 10 minutes. Reaction time should be adjusted for longer or shorter fragments. The reaction was quenched by adding 10 µl of allyl alcohol (Fisher). The mixture immediately turns yellow upon quenching. This color is due to the MnO_2 produced which does not affect the rest of the procedure. The mixture was lyophilized to dryness and piperidine cleavage was performed as described by Maxam and Gilbert (3).

C-Specific Modification by $NH_2OH \cdot HCl$

The $NH_2OH \cdot HCl$ (Mallinckrodt) reagent was prepared as a 4.02 M stock solution, adjusted to pH 6.0 with diethylamine (Aldrich). It is stable for at least one week if refrigerated.

A 7 µl aliquot of labelled DNA (roughly 15-20% of the total cpm in the sample) in water and containing 2-5 µg of unlabelled carrier DNA was denatured by heating at 90°C for 2 minutes followed by quick-chilling in ice water. Twenty microliters of 4.02 M $NH_2OH \cdot HCl$, pH 6, were mixed in and the solution was incubated at 20°C. The fragment shown in Figure 1 was 300 nt long and the incubation period yielding the best result was 10 minutes. Adjust reaction times for longer or shorter fragments. The reaction was diluted with 200 µl of 0.3 M sodium acetate, 0.1 mM EDTA and 3 µg of yeast tRNA. 95% ethanol was mixed in to a final volume of about 1.5 ml, the mixture was chilled at -70°C for 5 minutes, and the DNA was spun down in a

Beckman Microfuge for 5 minutes. The pellet was resuspended in 250 µl of 0.3 M sodium acetate, ethanol was again added to 1.5 ml and the precipitation was repeated. After centrifugation the pellet was rinsed with 1.5 ml of ethanol, centrifuged briefly, and the ethanol removed. The pellet was dried by lyophilization. Piperdine cleavage was as described by Maxam and Gilbert (3).

C-Specific Modification by $N_2H_4 \cdot CH_3COOH$

A sample was prepared for the hydrazine C-only cleavage according to the procedure of Maxam and Gilbert (3), except that 15 µl of ice-cold glacial acetic acid (Mallinckrodt) instead of 5 M NaCl were added to 35 µl of the hydrazine-DNA mixture. Optimum incubation times are slightly less than those given by Maxam and Gilbert (3). The reaction was ethanol precipitated and treated with piperdine as described by Maxam and Gilbert (3).

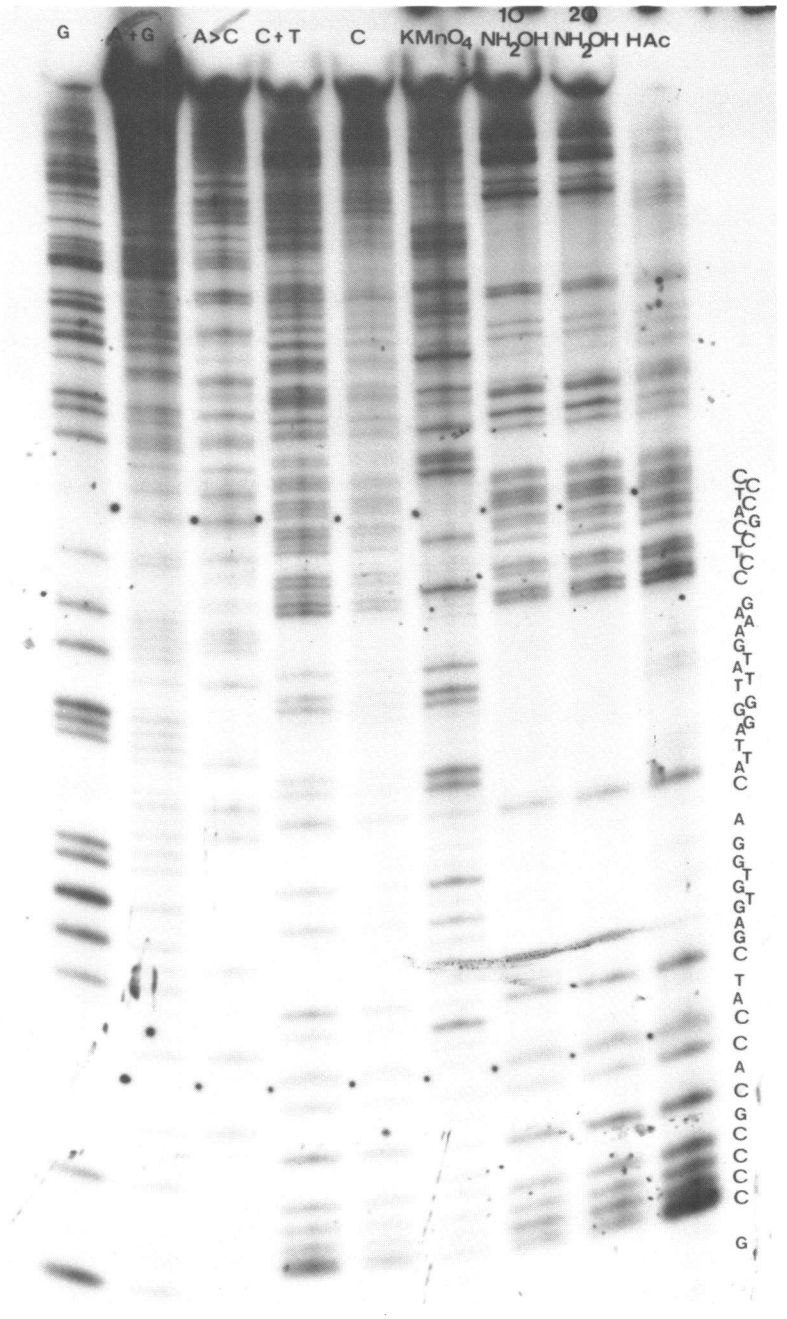
Samples were prepared for electrophoresis and loaded onto a 12% polyacrylamide sequencing gel. Following electrophoresis, the gel was quick-frozen between two slabs of dry ice and exposed onto Kodak XOMat film at -70°C for two weeks.

RESULTS

Figure 1 displays the $KMnO_4$ T reaction, the $NH_2OH \cdot HCl$ C reaction, and the $N_2H_4 \cdot CH_3COOH$ C reaction compared to the reactions described by Maxam and Gilbert (3). The sequence is a portion of a cloned human repetitive DNA (5, 6).

The reaction with $KMnO_4$ preferentially cleaves T with a faint background corresponding to cleavage at C and G. This result is consistent with the known reactivity of $KMnO_4$ toward nucleotides (see Discussion). It is also noteworthy that some T positions react more readily with $KMnO_4$ than others. Presumably, this variation is due to preferential cleavage of T at a particular primary or secondary structure (see Discussion).

The reaction with NH_2OH shows marked specificity for C (Figure 1). The background reactivity of hydroxylamine with other bases is comparable to the background in the Maxam-Gilbert C-specific reaction with hydrazine (Figure 1). The Maxam-Gilbert C-specific reaction with hydrazine employs concentrated NaCl to suppress the reactivity of hydrazine with T. We find that either concentrated acetic acid or sodium hydroxide is as effective as NaCl in suppressing the reaction of hydrazine with T.


G
A+G
A>C
C+T
C
KMnO4
10
NH2OH
20
NH2OH
HAc

DISCUSSION

$KMnO_4$ is a classic reagent for oxidation of double bonds in organic molecules, including nucleic acids and nucleosides (1, 7-11).

The proposed reaction mechanism is a straightforward glycolization of the 5-6 double bond in pyrimidines followed by oxidation to carboxylic acid and/or aldehyde products and ring opening (7, 11). The rate of reactivity of mononucleotides with $KMnO_4$ decreases as dT >> dC > dG, dA (1). We observe that though T is by far the base most often cleaved, a faint background is sometimes seen corresponding to C and G (Figure 1).

Hayatsu and Ukita previously demonstrated that T could be specifically oxidized by $KMnO_4$ (1). We have adapted this reaction to conditions suitable for Maxam-Gilbert sequencing (3).

Hayatsu and Ukita have also shown that reactivity with $KMnO_4$ depends upon the secondary structure of the DNA (1). Double-stranded DNA is attacked very slowly, even at high $KMnO_4$ concentrations, while single-stranded regions are readily modified under mild conditions (1). We have observed that some T positions in a strand are modified more readily than others, giving rise to dark and faint bands in the autoradiogram (Figure 1). This effect may be due to formation of some secondary structure within the denatured single strands.

We have purposely chosen to perform the reaction in unbuffered $KMnO_4$ for two reasons. First, very low ionic strength kinetically and thermodynamically favors base pair denaturation. Second, these conditions eliminate the need for the ethanol precipitations which Maxam and Gilbert use to desalt the DNA fragments after modification (3). It has been our experience that a significant amount of labelled DNA often fails to precipitate, even when carrier tRNA is added. Thus, the elimination of this step can result in a saving of time and labelled fragment.

The $KMnO_4$ T-only reaction is easier to read on a sequencing gel than

Figure 1. A sequencing gel comparing the standard Maxam-Gilbert chemical sequencing reactions with NH_2OH-modified C, $N_2H_4 \cdot CH_3COOH$-modified C and $KMnO_4$-modified T reactions. The base sequence is shown on the right, reading upwards from 3' to 5'. Lanes for N_2H_4-modified C and NH_2OH-modified C are indistinguishable. $KMnO_4$-modified T bands are of variable darkness, but the bases are still easily readable. The $N_2H_4 \cdot CH_3COOH$-modified C lane shows some T cleavage, but the difference in cleavage between C and T is so marked that it is easy to distinguish these bases from each other. Lanes are keyed as follows: (G), (A + G), (A > C), (C + T) and (C) are standard Maxam-Gilbert reactions (3). $KMnO_4$, permanganate T-only reaction; 10 NH_2OH, hydroxylamine C-only reaction incubated for 10 minutes; 20 NH_2OH, same as the previous lane except incubated for 20 minutes; HAc, hydrazine acetate C-only reaction. X = methyl cytosine.

the C + T reaction (Figure 1). A potential drawback to using the T only reaction alone is the variability in band strength mentioned above. For this reason, we chose to run both the $KMnO_4$ T-only reaction and the Maxam-Gilbert C + T reaction whenever possible.

Hydroxylamine is specific for C residues at pH 6 (2). While uracil (U) is quite reactive with NH_2OH at pH 10, T remains inert to this reagent at all pH's (2).

It has been proposed that NH_2OH adds across the 5-6 double bond in U and C, which then labilizes the ring to internal rearrangement and cleavage (2).

We prefer using $NH_2OH \cdot HCl$ to N_2H_4 in the C-only reaction. Degraded hydrazine may cause incomplete T supression even under high salt conditions (3). We also find that removal of the concentrated NaCl used in this reaction by ethanol precipitation is difficult, causing anomalous electrophoretic mobility and blurriness in the sequencing gel. In contrast to N_2H_4, $NH_2OH \cdot HCl$ is a stable solid which dissolves well in ethanol. However, we suggest that those who prefer to use N_2H_4 for C- specific reactions try $N_2H_4 \cdot CH_3COOH$ instead of N_2H_4 in 5 M NaCl, which Maxam and Gilbert specify (3) and decrease the reaction time slightly (Figure 1). $N_2H_4 \cdot CH_3COOH$ yields excellent C cleavage accompanied by a faint T background, but is more easily removed by ethanol precipitation than NaCl and N_2H_4. $N_2H_4 \cdot CH_3COOH$ could conceivably be removed by direct lyophilization to avoid ethanol precipitation, provided precautions are taken to trap and neutralize the hydrazine.

Investigators have had a choice of several purine-specific reactions for some time (3, 4). We do not necessarily recommend these two new reactions as substitutes for the standard Maxam-Gilbert reactions, but as alternatives. Ideally the new T- and C-specific reactions can be combined with the standard Maxam-Gilbert reactions to provide independent confirmatory readings.

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