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Adenine specific DNA chemical sequencing reaction

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ABSTRACT

Reaction of DNA with K_2PdCl_4 at pH 2.0 followed by a piperidine workup produces specific cleavage at adenine (A) residues. Product analysis revealed the K_2PdCl_4 reaction involves selective depurination at adenine, affording an excision reaction analogous to the other chemical DNA sequencing reactions. Adenine residues methylated at the exocyclic amine (N6) react with lower efficiency than unmethylated adenine in an identical sequence. This simple protocol specific for A may be a useful addition to current chemical sequencing reactions.

INTRODUCTION

DNA sequence determination according to the method of Maxam and Gilbert utilizes base specific chemical modification reactions followed by a workup which causes cleavage of the sugar-phosphodiester backbone at the site of the modified base.[1] To date, reactions have been reported which are capable of selective cleavage of DNA at G, G+A, A>G, A>C, C, C+T, and T residues[1-3]. We report here a chemical sequencing reaction for A that is simple and convenient. Reaction of DNA with K_2PdCl_4 at pH 2.0 followed by heating in the presence of piperidine produces an A specific DNA cleavage reaction.

MATERIALS AND METHODS

Preparation of Restriction Fragments

The 517 bp DNA fragment from plasmid pBR322 was prepared by literature methods with Eco R1 and Rsa 1 restriction endonucleases.[4] The 254 bp fragment of bacteriophage lambda DNA was prepared from N6-methyladenine free lambda DNA (λC1857Sam7 amplified in dam-,dcm-*E.coli*, Pharmacia) and lambda DNA (λC1857Sam7, Pharmacia) containing N6-methyladenine at 5'-GATC-3' sequences. In either case, 50μg of the DNA was digested with Sal 1 restriction endonuclease (New England Biolabs) and was then labeled at the 3' end with ^{32}P by incorporation of $\alpha^{32}P$-dCTP and $\alpha^{32}P$-dTTP using the standard Klenow fragment reaction.[2] The DNA was next digested with Xho 1 restriction endonuclease (New England Biolabs)

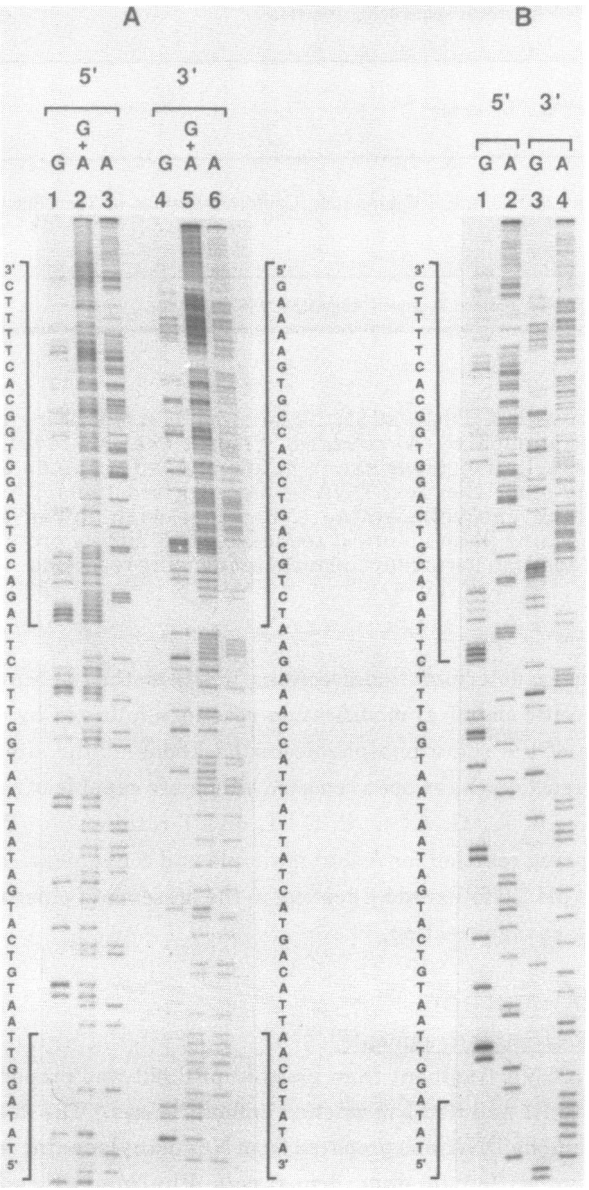

Figure 1. (A) Comparison of the G, G+A and A reactions on a 517 bp restriction fragment of DNA. Lanes 1-3 contain reactions on the 517 bp fragment labeled with ^{32}P at the 5' end. Lanes 4-6 contain reactions on the 517 fragment labeled with ^{32}P at the 3' end. Lanes 1 and 4, Maxam-Gilbert G reaction. Lanes 2 and 5, Maxam-Gilbert G+A reaction. Lanes 3 and 6, K_2PdCl_4 A reaction. (B) Comparison of G and A sequencing reaction on opposite strands. Each base position is represented by a band in only one of the four lanes illustrating a convenient sequencing strategy.

yielding a uniquely labeled 254 bp fragment which was isolated as the fastest moving radioactive band on a 5% nondenaturing polyacrylamide gel. After elution from the gel, the DNA was dialyzed against 1mM phosphate buffer (pH 7.3) prior to use.

Cleavage of DNA at Adenine with K_2PdCl_4

A 100mM HCl solution was adjusted to pH 2.0 with NaOH and potassium tetrachloropalladate(II), K_2PdCl_4, (Aldrich) was added to a final concentration of 10mM. 40µl of this solution was added to 160µl of a solution containing the ^{32}P labeled DNA fragment (5mR/hr) and 1µg of sonicated calf thymus DNA in distilled H_2O. The reaction was mixed and incubated at room temperature for 30-45 minutes and stopped by adding 50µl of a solution containing 1.5 M NaOAc, 1.0 M 2-mercaptoethanol and 20µg/ml of sonicated calf thymus DNA. 750µl of ethanol was added and the solution was chilled in dry ice for 10 minutes then spun at 12,000 rpm for 6-10 minutes. The supernatant was removed, the DNA pellet washed with 70% ethanol and dried briefly *in vacuo*. The pellet was redissolved in 50µl 10% aqueous piperidine, heated at 90°C for 30 minutes, frozen in dry ice and lyophilized. The lyophilized DNA was then dissolved in formamide buffer and loaded (0.5 mR/hr/lane) onto an 8% denaturing polyacrylamide (50% w/v urea) sequencing gel. After autoradiography, densitometry was performed using an LKB Ultroscan XL laser densitometer.

HPLC Analysis of the Reaction Products

5µl of 25mM K_2PdCl_4 was added to 50µg of calf thymus DNA (Sigma) in 50µl of H_2O. The solution was mixed and then incubated at room temperature for 3 hours. The reaction was terminated by the addition of 2µl of 2-mercaptoethanol then mixed, frozen, and lyophilized. In a control reaction, the 2-mercaptoethanol was added to the calf thymus DNA before the K_2PdCl_4 and the solution was immediately mixed, frozen and lyophilized. In each case, the lyophilized residue was dissolved in 8mM ammonium acetate (pH 5.5) and chromatographed on an Altex Ultrasphere ODS column eluted with a 30 minute linear gradient of 0-15% acetonitrile in 8mM ammonium acetate (pH 5.5) followed by a 15 minute linear gradient of 15-75% acetonitrile. The retention times of adenine and guanine were confirmed by coinjection with authentic samples.

RESULTS

A 517 bp restriction fragment of double-stranded DNA uniquely labeled at the 5' or 3' end was allowed to react with 2mM K_2PdCl_4 in 20mM HCl/NaCl pH 2.0 buffer at 25°C for 45 minutes followed by a piperidine workup. This produced an A specific sequencing lane (Fig. 1). The cleavage efficiency at each A is sufficiently

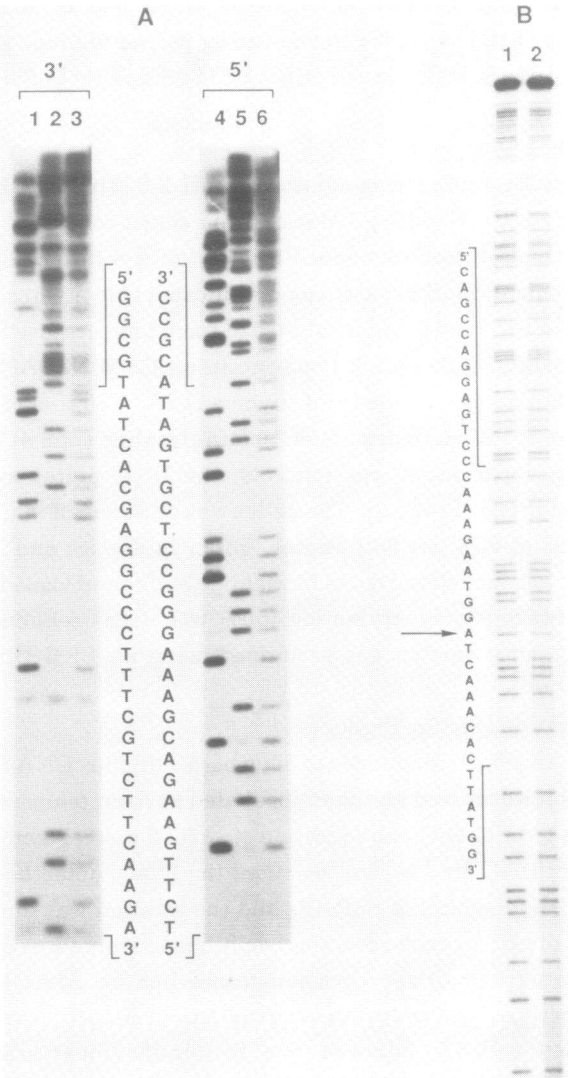

Figure 2. Gel A Comparison of the electrophoretic mobilities of the fragments produced by the G, A and G+A reactions on a high resolution gel (20% polyacrylamide). Lanes 1-3 contain reactions on the 517 bp fragment labeled at the 3' end. Lanes 4-6 contain reactions on the 517 fragment labeled at the 5' end. Lanes 1 and 4, Maxam-Gilbert G reaction. Lanes 2 and 5, K_2PdCl_4 A reaction. Lanes 3 and 6, Maxam-Gilbert G+A reaction. Gel B Comparison of the K_2PdCl_4 reaction on adenine and N6-methyladenine at a single location (arrow) in identical sequences of double stranded lambda DNA. Lane 1 contains the K_2PdCl_4 A reaction on a lambda DNA fragment with only unmethylated adenine. Lane 2 contains the K_2PdCl_4 reaction on the same lambda DNA fragment with N6-methyladenine at the location indicated by the arrow.

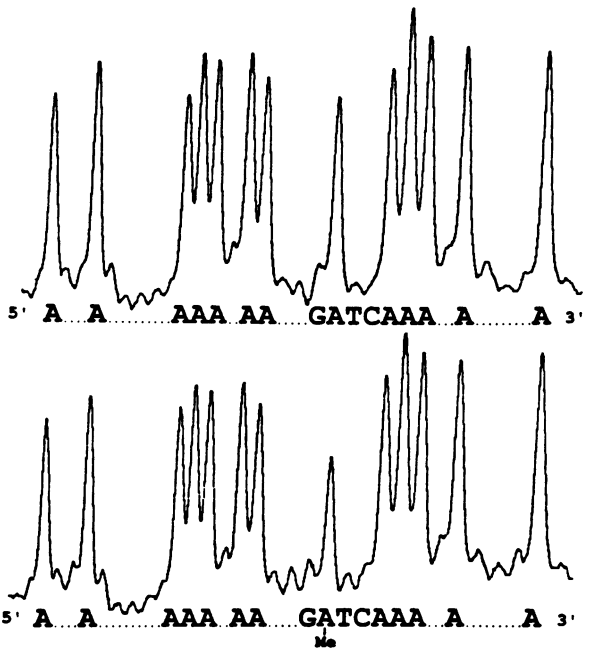

Figure 3. Densitometry of the middle region of in Figure 2B showing that the base position with N6-methyladenine (arrow, lane 2) reacts with a 35% lower efficiency than unmethylated adenine (lane 1) located in the same sequence.

uniform to enable unambiguous assignment of sequence. The electrophoretic mobilities of the fragments on a 20% polyacrylamide gel produced by the K_2PdCl_4 reaction are identical to the A cleavage fragments from the formic acid catalyzed G+A depurination reaction[2] which is known to produce 3' and 5' phosphate termini (Fig. 2A). The K_2PdCl_4 cleavage is therefore an excision reaction which can be used together with other base-specific reactions for complete DNA sequence determination. The K_2PdCl_4 reaction is stopped with thiol to coordinate the Pd(II), which might otherwise remain bound to the DNA and interfere with electrophoretic mobility. Standard G stop solution[2] can be used, so the A reaction is conveniently run in conjunction with the dimethylsulfate G reaction.[2]

HPLC analysis of the products of the K_2PdCl_4 reaction with calf thymus DNA revealed that the only apparent product released is adenine. Thus at low pH K_2PdCl_4 causes selective depurination of adenine residues.

In order to gain some information on the site of Pd(II) binding during depurination, the differential cleavage reactivity of N6-methyladenine and adenine residues was investigated. Two restriction fragments of lambda DNA were

prepared; one from lambda DNA amplified in a strain of *E. coli* which methylates the A in the sequence 5'-GATC-3', and an analogous fragment from lambda DNA amplified in a strain of *E.coli* (dam-, dcm-) which does not methylate the adenine residues. The reactivity toward Pd(II) at this adenine position was diminished by 35% in the restriction fragment containing the N6-methyladenine (Fig. 2B) when compared with the restriction fragment containing only unmethylated adenine (Fig. 3). Although other chemical sequencing methods have been reported which distinguish N6-methyladenine and adenine,[5-7] this reduction of cleavage at N6-methyladenine in the K_2PdCl_4 reaction might also be extensive enough to identify methylated sites if comparison with identical unmethylated sequences is available.

DISCUSSION

Spectroscopic studies with purine bases, nucleosides and nucleotides have indicated that Pd(II) binds the N7 and/or N1 positions.[8] For adenosine- (and guanosine-) 5'-monophosphate, Martin and coworkers have reported that the stability constants for (dien)Pd^{2+} binding to N1 and N7 are similar.[9] The stability constants for Pd(II) binding to guanine are higher than those for adenine.[9] The pK_a values for N1 and N7 in adenosine are 3.6 and -1.6, respectively.[8,10] Therefore, at low pH, Pd(II) binding to N7 should predominate, since metal binding must compete with protonation at the N1 position. The pK_a values for N1 and N7 of guanosine are 9.2 and 2.2, respectively, so at low pH, Pd(II) would be expected to bind N7 almost exclusively.[8]

The specific depurination of adenine by the reaction of DNA with K_2PdCl_4 at low pH could be produced by suppressing G reactivity, by enhancing specific depurination at A, or a combination of both. Since transition metals such as Pd(II) have been shown to bind N7 of guanine[8], we cannot rule out that the Pd(II) is binding to N7 of guanine and inhibiting to some extent the acid catalyzed depurination reaction at G.[2] However, we do find that the K_2PdCl_4 reaction at pH 2.0 causes cleavage at A with higher efficiency than occurs under the same pH conditions in the absence of K_2PdCl_4 suggesting that enhanced adenine depurination is occurring. Consistent with this, adenine is the major product released from the reaction of K_2PdCl_4 with calf thymus DNA at low pH. Presumably, the Pd(II) is binding to the adenine base in a manner which assists the hydrolysis of the glycosidic bond at low pH.

In a formal sense, the Pd(II) could bind to any of the nitrogen lone pairs on adenine; N1, N3, N7 or the exocyclic amine N6. Cleavage of DNA at sites containing N6-methyladenine was found to decrease by 35% when compared to unmethylated adenine in an identical sequence. The observed decrease in cleavage efficiency of

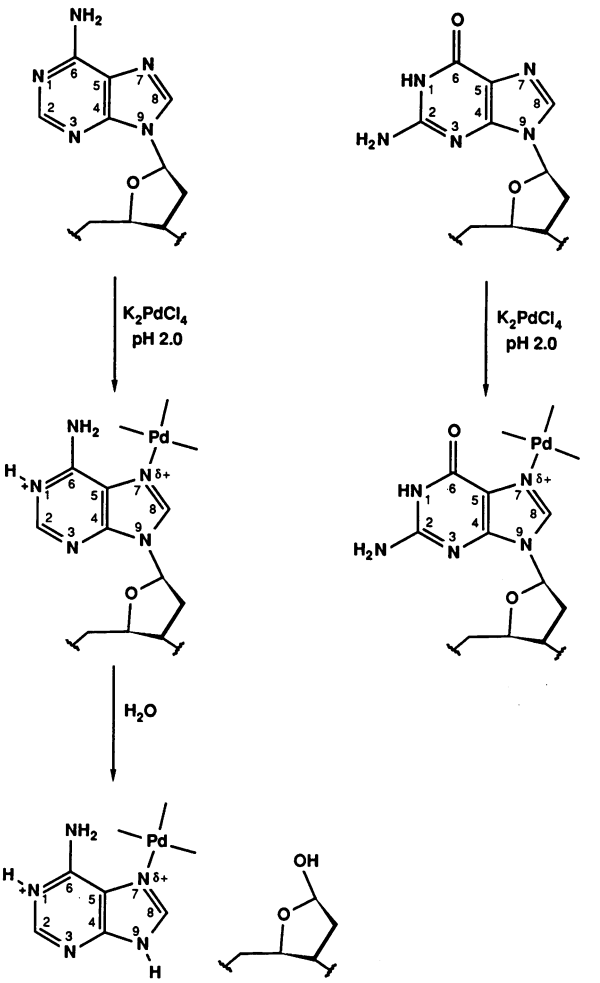

Figure 4. Proposed mechanistic scheme for the enhanced specific depurination of A≫G by K_2PdCl_4 at pH 2.0.

N6-methyladenine might be interpreted as resulting from the methyl group at N6 creating steric inhibition to binding by Pd(II) at N6, N1 or N7. Direct Pd(II) coordination to N6 is unlikely for Pd(II)[11] and N1 is expected to be protonated at pH 2.0. Therefore N7 emerges as the likely site of binding during the depurination reaction. The absence of significant neighboring base dependence on the observed A cleavage indicates that the depurination reaction caused by Pd(II) probably does not involve bridging bonds between N7 of adenine and adjacent bases.

An adenine bound by Pd(II) at N7 and protonated at N1 at pH 2.0 would contain a significant amount of positive charge on the adenine ring affording a labile glycosidic bond and allowing release of the adenine-Pd(II) complex (Fig. 4). Even though Pd(II) is probably binding to N7 of G, a similar depurination mechanism is not likely, since the protonated N1 position of G does not create a corresponding positive charge on the guanine ring (Fig. 4). This mechanistic scheme could be analogous to the Maxam-Gilbert reaction which involves dimethylsulfate alkylation of guanine (at N7) and adenine (at N3) followed by acid treatment (protonation at N1 of A) which results in selective depurination of A > G.[1]

In summary, the reaction of K_2PdCl_4 with DNA at low pH is a convenient and reliable method for the production of an adenine specific chemical sequencing lane. The reaction of K_2PdCl_4 with DNA at pH 2.0 probably involves binding of Pd(II) to N7 and protonation at N1 of adenine which results in specific depurination of adenine. The DNA is then cleaved at the depurinated sites by piperidine and heat. This A specific reaction may be timely with regard to developing chemistry for automated sequencing requiring four base specific chemical methods.

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