SPECIFIC SUPPRESSION OF DEPURINATION AT DEOXYGUANYLATE RESIDUES BY SILVER IONS

A useful reaction for the identification of deoxyadenylate residues in DNA

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The original form of the Maxam and Gilbert DNA sequencing method [1] utilized alkylation of DNA by dimethylsulphate followed by the kinetically rapid depurination of the polynucleotide chain at the positively charged 3-methyl deoxyadenylate residues as a specific reaction for the location of deoxyadenylate residues. Experience showing that this method is unreliable has led to its replacement by the less specific but more controllable acid depurination reaction [2]. This latter version of the sequencing method suffers from a minor disadvantage when compared to the primed synthesis methods [3,4] in that the deoxyadenylate residues must be identified by a comparison between 2 electrophoretic channels, of which one contains fragments cleaved only at deoxyguanylate residues and the other of which contains fragments cleaved at both deoxyguanylate and deoxyadenylate residues. Because of the high density of bands in the channel containing fragments cleaved at both types of purine nucleotide residue problems may arise in the interpretation of autoradiograms of DNA sequencing gels when long polypurine tracts are present. These problems are particularly severe when the sequence of interest does not lie close to a convenient site for end labelling. I describe here a simple and reliable modification to the conditions of the depurination reaction which confers a high degree of specificity for deoxyadenylate residues upon the reaction. This specific reaction depends upon the selective suppression of depurination at deoxyguanylate residues by high [Ag^+]. Details of this method are provided in fig.1.

The monovalent silver cation (Ag^+) has been widely used in studies of DNA base compositional heterogeneity to facilitate the separation of DNA species on the basis of differential buoyant density shifts in Cs_2SO_4 [5]. Although the primary sites of binding of this ligand to DNA have not been unambiguously identified, 2 discrete modes of binding have been distinguished [6,7]. Type I binding of Ag^+ to DNA is relatively insensitive to pH changes over the neutral range and does not involve the displacement of protons from the bases [6,7]. The most probable sites for type I binding are the nucleophilic nitrogen atoms at which protonation of the nucleotides occurs under acidic conditions (N-7 of deoxyguanylate, N-3 of deoxycytidylate and N-1 or N-7 of deoxyadenylate). Type II binding of Ag^+ to DNA is sensitive to pH changes and results in the displacement of H^+ from the DNA. Since the most readily displaced H^+ on DNA are bound at N-3 of thymidylate and N-1 of deoxyguanylate residues these nitrogen atoms are prime candidates for type II binding sites. The amino groups of adenine, guanine and cytosine are potential secondary type II binding sites.

Under the conditions of high [Ag^+] and low pH used here, the net decrease in the rate of depurination of pBR322 DNA (A/G = 1) due to the added Ag^+ is ~80% (see fig.2). Depurination must therefore be inhibited at both deoxyguanylate and deoxyadenylate residues. The hydrolysis of *N*-glycoside bonds in DNA proceeds via a unimolecular reaction and that the rate of hydrolysis is greatly enhanced by protonation of the base [8]. The rate of reaction for any individual nucleotide therefore depends upon the average electron density at the glycosidic nitrogen. Providing the type I site for silver binding on deoxyadenylate residues is the same as the site of protonation (N-1) the suppression of depurination at these

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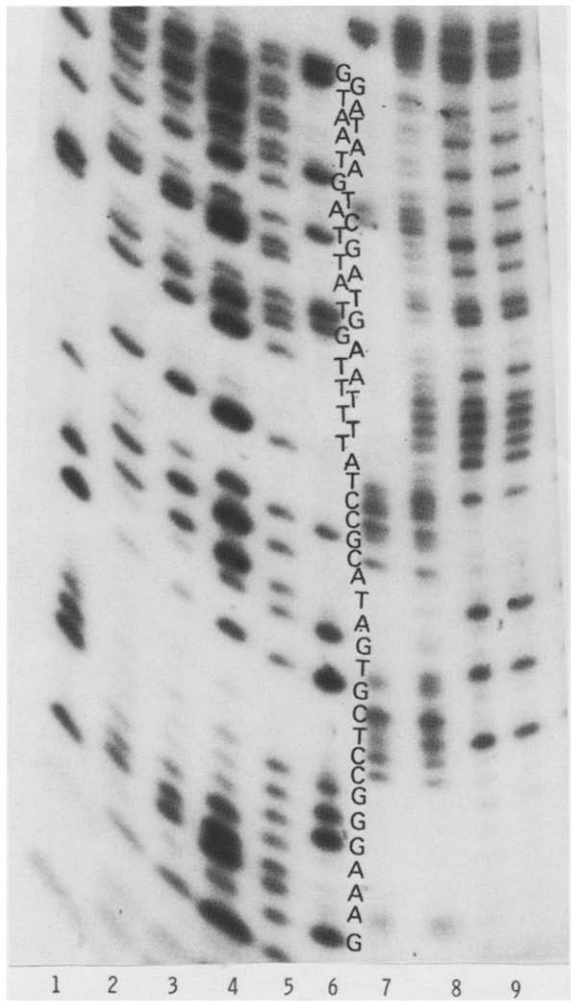

Fig.1. pBR322 DNA 5'-end labelled with [γ-^{32}P]ATP and polynucleotide kinase at the *Eco*RI site was re-cleaved with *Bam*HI into a large fragment carrying the penicillinase gene and a small fragment containing part of the tetracycline resistance gene. The fragments were separated by sucrose gradient centrifugation and the large fragment was subjected to sequence analysis: (1,6) guanine-specific cleavage according to [2]; (5) (adenine + guanine)-specific cleavage [2]; (7) cytosine-specific cleavage [2]; (8) (cytosine + thymine)-specific cleavage [2]; (9) thymine-specific cleavage [12] end-labelled DNA was precipitated with ethanol and redissolved in 25 μl 10 mM NaOH, 1 mM $NaIO_4$, 50 μM $KMnO_4$ and incubated at 23°C for 10 min. The reaction was terminated by the addition of 5 μl 1 M Na-acetate, 1 M β-mercaptoethanol and 100 μl ethanol. The precipitated DNA was then treated with piperidine as below for the adenine-specific cleavage reaction. (2–4) Adenine-specific cleavage: 2.5 × 10^5 dpm of the end-labelled fragment was ethanol precipitated and redissolved in 50 μl 0.2 M $NaNO_3$. At zero time 50 μl 0.02 M HNO_3, 1 mM $AgNO_3$ was added and the sample was incubated at 37°C. Aliquots (30 μl) were removed (2) 15 min, (3) 30 min and (4) 60 min, centrifuged at 11 000 rev./min for 5 min and the supernatants were aspirated. The acid-precipitated DNA samples were rinsed with 50 μl ethanol containing 10 mM β-mercaptoethanol and were then cleaved with 100 μl 1 M piperidine at 90°C for 30 min. Samples were lyophilized and applied to a thin 8% acrylamide sequencing gel as in [2]. The DNA sequence inferred from an examination of the autoradiogram is as in [13] for the corresponding region of the pBR322 genome.

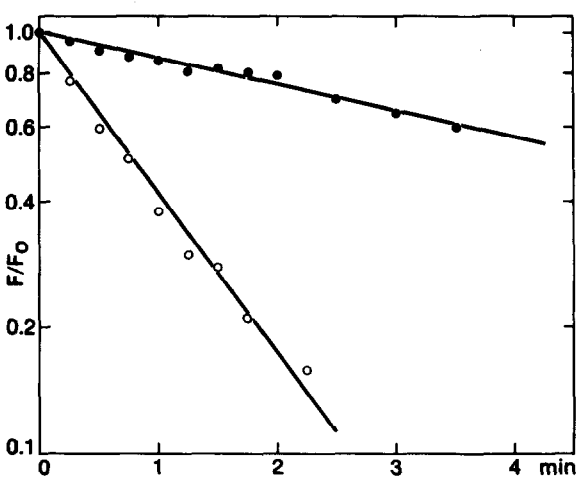

Fig.2. pBR322 DNA (5 μg) in 100 μl 0.2 M $NaNO_3$ was mixed with (A) 100 μl 0.02 M HNO_3; (B) 100 μl 0.02 M HNO_3 containing 2 mM $AgNO_3$ at 25°C. At the time points indicated 20 μl aliquots were taken and diluted into 1 ml 0.02 M trisodium phosphate (pH 12.0), 1 mM EDTA, 10 mM β-mercaptoethanol 0.5 μg ethidium bromide/ml [14]. Samples were heated to 95°C for 10 min to cleave depurinated sites and to denature nicked DNA. Residual enhancement of ethidium fluorescence which is due to remaining closed circular DNA molecules was determined after cooling the samples to 25°C. A further 10 min heat cycle did not result in any further changes in the fluorescences of the samples indicating that cleavage at apurinic sites was complete. The calculated pseudo first-order rate constants for the depurination reaction in the absence of added metal ligand is 3 × 10^{-2} s^{-1} under the above conditions. In the presence of 1 mM $AgNO_3$ the average rate constant is 4.7 × 10^{-3} s^{-1}. Note that these rate constants do not discriminate between the contributions of depurination at deoxyadenylate and deoxyguanylate residues. In the absence of metal ligand the rate constants are about equal while in the presence of 1 mM $AgNO_3$ depurination of deoxyadenylate residues is about 10-times more rapid than at deoxyguanylate residues.

residues results from direct competition between H^+ and Ag^+ for this site and the net positive change that is transferred to the base from a bound Ag^+ must be less than that transferred from H^+. The nitrogen–silver bond must therefore be highly polarized. Similar polarization of a bond between Ag^+ and N-1 (type II site) of a deoxyguanylate residue would leave a partial negative charge on the purine ring system. Although this partial charge would be reduced by the binding of a second Ag^+ to the type I site the net effect would be to reduce the rate of depurination at deoxyguanylate residues and to give the overall reaction the observed specificity for deoxyadenylate residues.

Cu^{2+} and Hg^{2+} bind strongly to DNA with affinities that are comparable to those observed for Ag^+. I have therefore used the same methods to examine the effects of Cu^{2+} and Hg^{2+} on the specificity and rate of the depurination reaction. Although Cu^{2+} (1 mM) cause a large reduction in the average rate of depurination, DNA sequencing gels showed that this ion suppressed depurination only slightly more effectively at deoxyguanylate residues than at deoxyadenylate residues. This observation is consistent with the mechanism discussed above since the additional positive charge on the Cu^{2+} would offset the partial negative charge induced in deoxyguanylate residues by type II binding of the metal ligand. Although in [9] Hg^{2+} bound strongly to DNA under acidic conditions, I observed no decrease in the rate of depurination of DNA when this ligand was added to the reaction mixture. Furthermore, when end-labelled DNA was subjected to the same treatment followed by treatment with piperidine, significant levels of cleavage at the pyrimidine residues were observed. Since Hg^{2+} alone does not promote high levels of depurination direct competition between Hg^{2+} and H^+ cannot explain the above result. Neither can this result be explained in terms of the simple model proposed for the case of Ag^+. Ag^+ and Cu^{2+} bound preferentially to AT-rich regions in DNA while Hg^{2+} binds preferentially to GC-rich regions [5,11]. Hg^{2+}, unlike Ag^+ or Cu^{2+}, releases 2 H^+ when bound in a 1:1 complex with deoxyadenylate [9,10]. These observations and those reported here suggest that the mode of binding of Hg^{2+} to DNA is fundamentally different from that of Ag^+ or Cu^+.

In addition to providing a useful cleavage reaction for the determination of deoxynucleotide sequence, this work illustrates a novel approach to the study of interactions between polynucleotides and metal ligands. The demonstration that Ag^+ imparts specificity to the acid depurination reaction suggests the general utility of metal ligands as modifiers of the chemical reactivity of polynucleotides.

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