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SEQUENCE ANALYSIS OF POLYRIBONUCLEOTIDES BY CONTINUOUS DIRECTIONAL DEGRADATION – A STUDY OF THE COURSE OF THE REACTION AT NUCLEOTIDE CONCENTRATIONS BETWEEN 10^{-7} AND 10^{-5} MOLAR*

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1. Introduction

As recently shown [1], incubation of polyribonucleotides in the presence of *E. coli* alkaline phosphophosphomonoesterase (EC 3.1.3.1) and $NaIO_4$ at pH 8.0–8.4 results in a $3' \rightarrow 5'$ directional degradation process in which dialdehyde derivatives are released sequentially from the polynucleotide chain. A sensitive assay of these compounds is based on reduction with [^{3}H]KBH_4 to labeled alcohols [1]. Analysis of the time course of the degradation process provides a means to deduce the sequence of the polynucleotide. According to the postulated mechanism [1], degradation is the result of a combination of several consecutive reactions: (i) oxidation of 3′-termini; (ii) β-elimination of esterified phosphate; (iii) enzymic hydrolysis of phosphomonoester bonds; (iv) secondary reactions of dialdehyde derivatives. In the reported studies [1], the nucleotide concentration was 10^{-3} M. We have now carried out kinetic studies on the basis of which we are able to deduce conditions enabling one to carry out this degradation and analyse its time course at extremely low nucleotide concentration (10^{-7}–10^{-5} M). The kinetic studies will be described elsewhere. Here we wish to report conditions for small amounts of polynucleotide. The results will demonstrate that the novel degradation procedure, when combined with tritium post-labeling, provides an extremely sensitive means for sequence analysis; in the case of a trinucleotide, as little as 0.001 A_{260} unit of nucleotide, in a volume of 100 μl (approx. concentration 3×10^{-7} M), suffices to establish the sequence.

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2. Experimental

2.1 *Degradation*

Except for the experiment depicted in fig. 3A, concentrations of nucleotide and reagents were 150–3 000-fold lower than in previously reported experiments [1]. ApUpG was incubated at 50°C in the presence of borate buffer (0.03 M, added buffer being 0.1 M $Na_2B_4O_7$/HCl, pH 8.45 at 23°C), $NaIO_4$, and alkaline phosphatase (Worthington code BAPF, ribonuclease-free). Reactions were carried out in the presence of tritium-labeled ribothymidine trialcohol ([^{3}H]T′) as an internal standard; in some cases internal standard was added after labeling (see below). Aliquots were withdrawn during the reaction and stored at –72°C until reduction with [^{3}H]KBH_4.

2.2 *Reduction of dialdehydes*

[^{3}H]KBH_4 treatment of samples from incubation mixtures containing 10^{-3} M ApUpG was carried out as described [1]. At lower concentration, reduction was initiated by adding a 10–15-fold molar excess of [^{3}H]KBH_4 (final concentration 5×10^{-4} M) in 0.1 N KOH over original $NaIO_4$ to aliquots from incubation mixtures containing 7×10^{-6} M ApUpG; at the lowest nucleotide concentrations investigated (10^{-6} M and 3×10^{-7} M), excess of [^{3}H]KBH_4 was 20–25-fold (final concentration 2×10^{-4} M). Specific activity of [^{3}H]KBH_4 was 2.5 Ci/mmole, except

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for the 10^{-3} M experiment where it was 0.2 Ci/mmole.

Reduction was carried out usually for 2 hr at 23°C in the dark (4 hr at 3×10^{-7} M ApUpG). Excess [^{3}H] KBH_4 was destroyed by adding a 3–5-fold molar excess of 1 N acetic acid over borate, and the solution was taken to dryness in a stream of air [2]. It was then immediately subjected to two-dimensional silica gel thin-layer chromatography and quantitative analysis [1].

2.3 *Preparation of internal standard*

This was done as reported for tritium-labeled nucleoside trialcohols [2]. The final solution (in water) contained 0.4 nmole [^{3}H] T′ (0.46 μCi)/μl. In experiments where original nucleotide concentration was 7×10^{-6} M, 1 μl of solution of internal standard was added per 100 μl of original reaction mixture. Under more dilute conditions, internal standard was added after reduction when the final dried residues were taken up in dilute ($2–4 \times 10^{-6}$ M) aqueous solution of [^{3}H] T′ rather than water.

3. Results and discussion

3.1 *Chromatography*

Fig. 1 illustrates a two-dimensional silica gel map of nucleoside trialcohols (N′)* and nucleoside methylene dialcohols (N″)**.

3.2 *Influence of enzyme concentration*

Fig. 2 shows dependence of the overall reaction on phosphatase concentration at 7×10^{-6} M nucleotide. Initial rates of release of dialdehydes originating from non-3′-terminal positions can be seen to depend strongly on enzyme concentration whereas formation of the derivative from the 3′-terminus itself is independent of phosphatase concentration. This is to be expected since, in accordance with the postulated mechanism [1], for 3′-dephosphorylated polynucleotides, only release of internal and 5′-terminal positions requires action of phosphatase. Fig. 2 demonstrates also that individual peak heights (and hence sensitivity of the method) are determined by enzyme concentration. This observation also applies mainly to peaks derived from non-3′-terminal positions. Peak heights are defined by relative rates of two competing consecutive reactions: β-elimination leading to formation of nucleoside methylene dialdehydes and decomposition of dialdehydes as a result of secondary reactions. According to the postulated reaction mechanism [1], rate of β-elimination is expected to depend on rate of removal of 3′-terminal phosphate, which in turn depends on enzyme concentration at low enzyme/substrate ratios. Higher enzyme concentrations thus favor rapid formation and accumulation of appreciable amounts of dialdehyde derivatives from non-3′-terminal positions in the reaction mixture, as shown in fig. 2.

* N′ (A′, C′, U′, G′), a nucleoside trialcohol.
** N″ (A″, C″, U″, G″), a nucleoside methylene dialcohol.

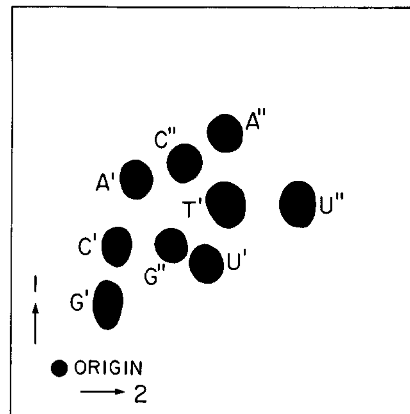

Fig. 1. Two dimensional silica gel TLC of nucleoside trialcohols and nucleoside methylene dialcohols: First dimension, acetonitrile/15 N ammonia (3.4 : 1) to 15 cm; second dimension, acetonitrile/water/90% (w/w) formic acid (11 : 1 : 0.3) to 15 cm, see [1].

3.3 *Influence of nucleotide and $NaIO_4$ concentrations*

Results presented in fig. 3 reveal a similar course of the reaction over a wide range of concentrations of nucleotide, phosphatase and periodate. Peak heights on panels A, B, and C of this figure are not directly comparable since in experiment A [^{3}H] KBH_4 of low specific activity was used [1] and final labeled solutions contained a different amount of internal standard relative to original ApUpG (see above); fig. 3C is, however, directly comparable to figs. 2A–C.

Although experiments A and B (fig. 3) show a striking overall similarity of the course of the reaction,

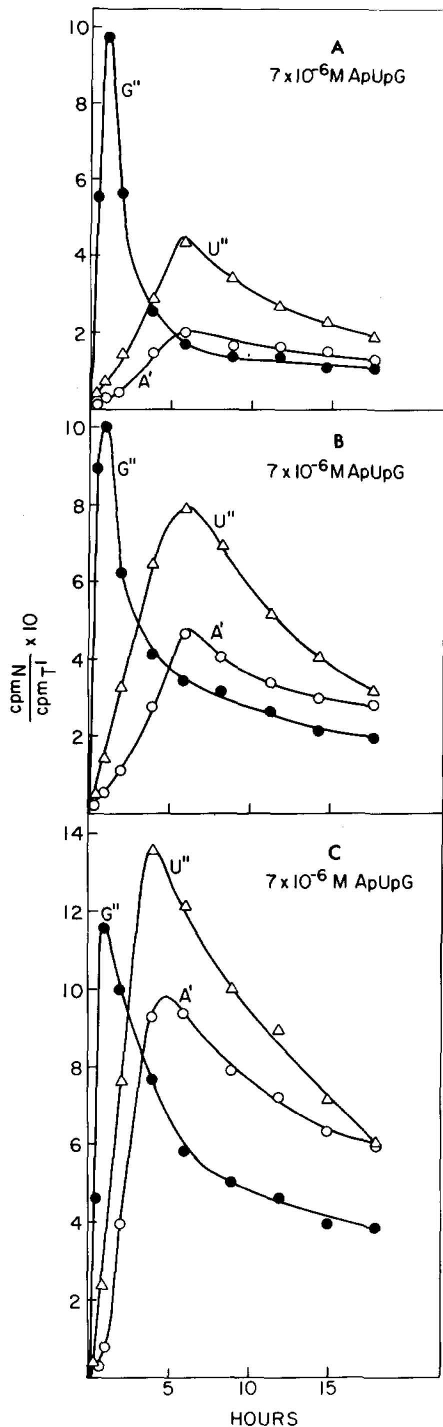

Fig. 2. Influence of phosphatase on kinetics of degradation. All incubation mixtures contained ApUpG (7×10^{-6} M), borate (3×10^{-2} M, see text, $NaIO_4$ (4.5×10^{-5} M), and alkaline phosphatase (0.001 μg/μl in expt. A, 0.003 μg/μl in expt. B, 0.013 μg/μl in expt. C). Incubation was at 50°C in the dark. Aliquots were removed from the mixture at indicated times and analyzed by tritium labeling and chromatography, see text.

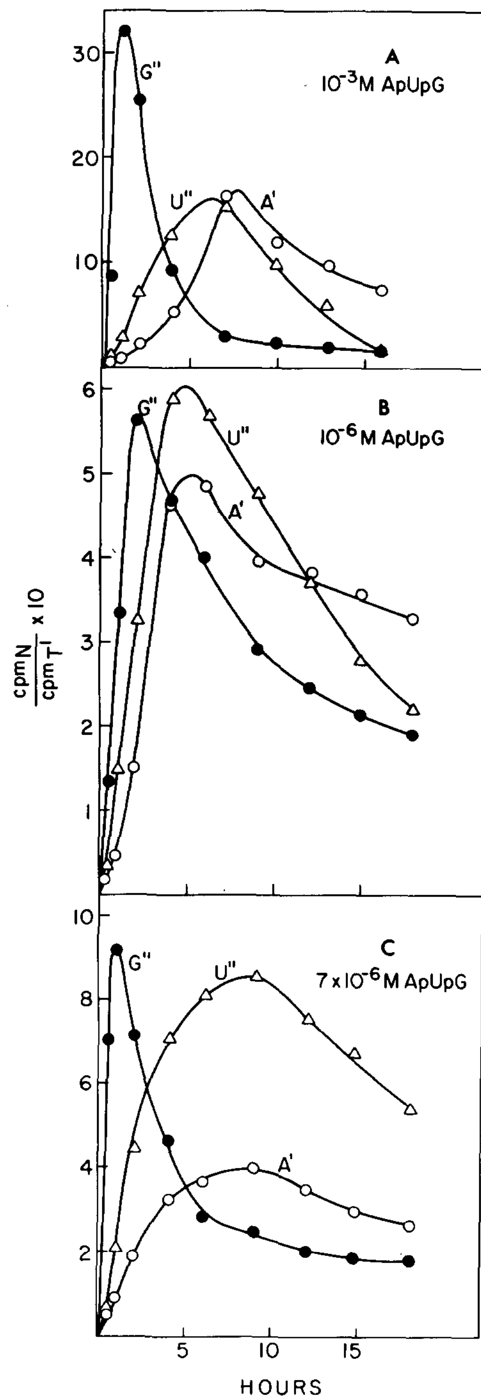

Fig. 3. Course of reaction at different nucleotide and periodate concentrations. All solutions contained 3×10^{-2} M borate, see text. The initial concentrations were: 10^{-3} M ApUpG, 6×10^{-3} M $NaIO_4$, 0.2 μg/μl phosphatase (expt. A); 10^{-6} M ApUpG, 10^{-5} M $NaIO_4$, 0.0044 μg/μl phosphatase (expt. B); 7×10^{-6} M ApUpG, 1.5×10^{-4} M $NaIO_4$ 0.013 μg/μl phosphatase (expt. C). For conditions of incubation and analysis, consult legend of fig. 2 and text.

in spite of a 1 000-fold difference in nucleotide concentration, there are some distinct differences: (i) the peak derived from the 3′-terminus (G″) occurs later at 10^{-6} M than at 10^{-3} M concentration of nucleotide, and the initial reaction rate is slower; (ii) peaks derived from second (U″) and third (A′) positions, on the other hand, occur earlier at lower nucleotide concentration, and rates of formation of the parent dialdehydes are faster; (iii) relative peak heights are different in the two experiments.

On the basis of kinetic studies (unpublished), these observations may be explained as follows: (i) slower formation of the G″ peak in experiment B is not the result of a decreased rate of β-elimination since this reaction, at constant pH, follows first-order kinetics, half-life of nucleotide dialdehyde being independent of its initial concentration (about 40 min at pH 8.45 and 50°C). Rate of oxidation, on the other hand, is of the second order at low concentration [3,4], depending on both nucleotide and periodate concentration so that 3′-terminal oxidation becomes rate-determining whereas at the higher concentration (fig. 3A) β-elimination is rate-limiting, oxidation being complete within 1–2 min; (ii) earlier appearance, in experiment B, of peaks derived from non-3′-terminal positions is to be attributed to a 20 times higher phosphatase/nucleotide ratio as compared with experiment A; (iii) different peak heights are likewise the result of different enzyme/substrate ratios in the two experiments. At still higher enzyme/substrate ratios, dephosphorylation follows first-order kinetics with respect to nucleotide, but this situation has not been completely realized in these experiments.

As shown by a comparison of figs. 3C and 2C, an increase in $NaIO_4$/nucleotide ratio results in diminution of all peaks. Studies, to be reported elsewhere, have shown that at high $NaIO_4$ concentrations and high $NaIO_4$/nucleotide ratios, overoxidation of dialdehydes occurs leading to formation of some free base. It appears that most or all changes seen at high $NaIO_4$ /nucleotide ratios can be attributed to overoxidation.

Reduction of nucleotide concentration, in our experience, requires a compensatory raise in both $NaIO_4$/nucleotide and enzyme/nucleotide ratios in order to maintain similar reaction rates. For example, the course of the reaction at 3×10^{-7} M ApUpG is similar to the one at 10^{-6} M (fig. 3B) if both ratios are increased three-fold (initial concentrations: 3×10^{-7} M ApUpG, 10^{-5} M $NaIO_4$, 0.0044 μg/μl phosphatase).

The degradation reaction is applicable to polynucleotides of higher molecular weight such as tRNA; under conditions similar to those described here (unpublished), we were able to identify the first six positions from the 3′-terminus of $tRNA^{Tyr\,2}_{E.coli\,B}$ (= CpCpApCpCpA [5]).

It appears likely that, under controlled conditions, one will be able to calculate precisely and predict the kinetics of degradation of relatively long oligonucleotides. It should thus be possible to establish the sequence of these compounds solely on the basis of careful analysis of the early course of the reaction. This question is currently being investigated.

Acknowledgements

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