The Occurrence of Autoacceleration during Hydrolysis of Nucleic Acids by Micrococcal Nuclease*

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SUMMARY

In the course of digestion of nucleic acids by micrococcal nuclease in the presence of low Ca++ concentration, three distinct phases of the reaction are observed. The first and the third are slow, and the second is fast. The reaction rate during the first phase is proportional to Ca++ concentration. An autoaccelerated second phase, showing little dependence on Ca++ concentration, has been observed with native DNA, denatured DNA, and RNA. The apparent end point (termination of the third phase) occurs earlier with decreasing Ca++ concentration.

Of the three substrates studied, native DNA, RNA, and denatured DNA, native DNA is the most resistant. However, since each has a different requirement for Ca++, the relative hydrolytic rates vary widely with Ca++ concentration. Introduction of 0.15 M NaCl into the reaction medium accelerates the first phase and decelerates the second phase. The over-all reaction is slower, and the extent of hydrolysis is decreased.

Under identical conditions, micrococcal nuclease hydrolyzes denatured DNA much faster than native DNA. However, in the presence of both substrates, it does not hydrolyze denatured DNA exclusively.

The autoacceleration phenomenon suggests that the best substrate for micrococcal nuclease is a polynucleotide chain of intermediate length (between nucleic acids and short oligonucleotides), and, therefore, the enzyme should be classified as a polynucleotidase.

No endonuclease is known with an absolute specificity toward either native or denatured DNA. However, a great majority of nucleases show a significant preference, ranging in magnitude from several-fold to many thousand fold, toward double or monostranded form (1). As a consequence, it may be expected that kinetics of hydrolytic degradation of DNA will be complicated by the effect of conformational change. A nuclease that more readily attacks the double-stranded form will show autoretardation, because the double-stranded structure does not persist for long. The word autoretardation was coined to characterize kinetic changes observed with DNase I (2). Even more pronounced changes of a similar nature have been previously described with DNase II (3).

An opposite effect, autoacceleration, was first observed by Jorgensen and Koerner (4), who discovered an enzyme originally called oligonucleotidase, later named Escherichia coli exonuclease IV. Autoacceleration could also be expected with micrococcal nuclease because numerous authors (5-9) reported that this enzyme shows preference toward denatured DNA, even though the quantitative evaluation varies from 2-fold (8) to 100-fold (5) with several intermediate values (6, 7, 9).

In view of the findings of Jorgensen and Koerner (4), it seems plausible that both changes in conformation and in chain length of the substrate affect the reaction rate, although both factors are partially dependent on each other. This contention is indirectly supported by observations of many authors (5, 6, 10-12) that micrococcal nuclease shows preference for the Np—Ap bond and Np—1p bond as long as it attacks native DNA. The cleavages become random (7, 9, 11, 12) when denatured DNA serves as substrate, suggesting that in the helical form the Np—Cp and Np—Gp linkages are relatively resistant.

Moreover, in the early stages of reaction, micrococcal nuclease is a typical endonuclease. As the reaction progresses, exonucleolytic cleavages gradually increase, and on the level of a trinucleotide, only the Np³ nucleotide is liberated (13). The transition from endo- to exonucleolytic character may and probably does reflect the rate of reaction.

This paper describes experiments that show the auto-

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accelerated phase of reaction. The effects of calcium and the nature of substrate on this phenomenon are described.

EXPERIMENTAL PROCEDURE

Materials—Thymus DNA was prepared in this laboratory according to the procedure of Kay, Simmons, and Dounce (14). An aqueous solution of DNA (1 mg per ml) was prepared by gentle stirring in a cold room for 72 hours. The solution contained 20 A260 units per ml. It is referred to as "native DNA," even though it was recently shown to contain about 2.5% of denatured DNA (15). Heat-denatured DNA was prepared by heating an aqueous solution of DNA (1 mg per ml) at 100° for 30 min and rapid cooling on ice. Yeast ribonucleic acid, prepared according to the method of Crestfield, Smith, and Allen (16), was purchased from Worthington Biochemical Corporation (Freehold, New Jersey). The aqueous solution of RNA (1 mg per ml) contained 17 A260 units per ml. Bovine albumin (crystallized) was obtained from Pentex. Crystalline, phosphatase-free micrococcal nuclease was prepared by a previously published procedure (17). All chemicals used were of reagent grade. Water was distilled three times, the last two times from a quartz apparatus.

Methods—Enzyme activity measurements were performed spectrophotometrically, essentially as described by Cuatrecasas et al. (8), at 37° with a Gilford recorder. The assay mixture consisted of 4 A260 units of DNA, 0.05 M triethylamine acetate (pH 9.0), 2.5 mM CaCl2, and enzyme, in a total volume of 4 ml. One unit of activity was defined as a change in absorbance at 260 nm of 1.0 per min. Use of the spectrophotometric method was limited to establishing the activity of the enzymes. All other measurements were performed in a pH-stat.

Hydrolysis was followed with the use of the pH-stat described by Peanasky and Szucs (18). The NaOH storage and delivery systems were as described by Richards et al. (19). A steady stream of water-saturated nitrogen was passed over the incubation mixture kept in a water-jacketed cell at 37°. All enzymatic reactions were effected at pH 9.0 with 0.01 N NaOH as titrant. The final volume of reaction mixture, regardless of its composition, was always 4 ml. The reaction mixture was preincubated for 20 min before adjusting with 0.01 N NaOH to pH 9.0. The enzymatic reaction was then started by an addition of 10 μl of micrococcal nuclease. The stock solution of micrococcal nuclease was diluted to a desired concentration with 0.1% solution of serum albumin and kept on ice. Under these conditions, the enzyme retained activity for 10 days. Freezing and thawing of enzyme were avoided. The consumption of NaOH during hydrolysis was corrected for the base line of NaOH uptake established in control experiments. In all experiments in which NaCl was used, the concentration was 0.15 M. Variations in the amounts of substrate, enzyme, and CaCl2 in individual experiments are specified in the figure legends.

RESULTS

From the time of discovery of micrococcal nuclease by Cunningham, Catlin, and Privat de Garille (20), the majority of workers have used their value of 10 mM Ca++ as optimal. Cunningham et al. (20) and Dirksen and Dekker (5) found that Ca++ concentrations higher than 10 mM were inhibitory. They also observed that addition of NaCl to the reaction medium markedly decreased the rate of the reaction. Fig. 1 illustrates the results of experiments in which native DNA and high Ca++ concentrations ranging from 10 mM to 50 mM were used. These experiments confirm the previous observations (5, 20) in both respects. In view of what follows, attention is now called to the fact that superoptimal Ca++ concentrations inhibit the entire course of the reaction.

Fig. 2 illustrates the results of similar experiments except that
the Ca++ concentrations are suboptimal and vary from 75 μM to 600 μM. These experiments show that the reaction consists of three phases. The first and third are slow, while the second is fast. At 500 μM Ca++ (the highest shown in Fig. 2), Phase 1 is barely discernible. With this Ca++ concentration, the rate of Phase 1 approaches that of Phase 2. Tangents drawn through the midpoints of curves representing Phase 2 show almost identical slopes, suggesting that at low Ca++ concentrations the rate of the second phase of the reaction is relatively independent of Ca++ concentration. This must be contrasted with high Ca++ concentrations (Fig. 1) which delineate the inhibitory effects of Ca++ throughout the whole course of the reaction.

The graph (not shown) representing a plot of Ca++ concentration with respect to rate of Phase 2 shows a plateau in the range from 0.1 mM to 10 mM Ca++, but a definite and continuous decrease as the Ca++ concentration increases beyond 10 mM. Presumably, the ascending arm of the curve lies in the region 0 to 2.5 μM (see Fig. 3) which could only be investigated with reagents of unusual purity with respect to Ca++ contamination.

Fig. 2 shows another interesting phenomenon, namely, that the introduction of 0.15 m NaCl abolishes the triphasic nature of the reaction, with a concomitant return to normal kinetics. The early part of the reaction is accelerated and the latter part retarded by NaCl. This observation explains the discrepancies encountered in the literature concerning the extent of inhibition by NaCl (5-9).

The experiments represented by Fig. 3 were performed with denatured DNA and the same amount of enzyme as the experiments of Figs. 1 and 2. However, to slow down the reaction, Ca++ concentrations had to be limited to the range from 2.5 μM to 50 μM. Autoacceleration is clearly visible in Fig. 3, as it is in Fig. 2. Similarly, autoacceleration was abolished by 0.15 m NaCl, with stimulation of the early phase of the reaction and depression of the later phase. It is clearly seen in both Figs. 2 and 3 that the end point of the reaction is dependent on Ca++ concentration.

Experiments shown in Fig. 4 were performed with denatured DNA and Ca++ concentrations in the range from 0.1 mM to 10 mM, but with only 0.05 unit of enzyme, one-fifth of the amount previously used. Hydrolysis of denatured DNA is not inhibited by NaCl at high Ca++ concentrations except for the last stage of reaction. In contrast, hydrolysis of native DNA is inhibited throughout the entire reaction (Fig. 1).

Fig. 5 shows the results of experiments with RNA, 0.25 unit of enzyme, and Ca++ concentrations from 10 μM to 50 mM. For graphic clarity, the experiments without NaCl are shown in Fig. 5A; those with NaCl are shown in Fig. 5B. Autoacceleration is observed in Experiment 2 of Fig. 5A. Addition of NaCl decreases the rate throughout the whole course of the reaction for all Ca++ concentrations used.

Fig. 6 shows the results of experiments in which Ca++ concentration was constant at 2.5 mM but the amount of enzyme varied from 0.025 unit to 0.25 unit, with either native DNA (— — —) or RNA (— — —) as substrate. At all the levels of enzyme concentration, RNA is digested faster than DNA during the early part of the reaction. This is particularly evident with the lowest level of enzyme (Experiment 1). At later stages of the
reaction, DNA is digested faster. This observation supplies a plausible explanation for the discrepancies existing in the literature (8, 10) with respect to preference of the enzyme toward the sugar moiety of the substrate.

Fig. 7 shows the dependence of the initial rate of hydrolysis on Ca++ concentration for the three substrates used. Native DNA and RNA show an optimum at about 10 μM in agreement with

Fig. 7 shows that the reaction with denatured DNA is complete in 8 min, whereas the accelerated phase with native DNA begins after about 25 min. It seemed warranted to utilize this observation for probing DNA conformation with the enzyme. Fig. 8 shows that the difference in the rate of hydrolysis of native and denatured DNA both at 100 μM Ca++ is striking. Mixing the two substrates in different proportions produces a series of intermediate curves.

A comparison of Experiment 2 of Fig. 2 with Experiment 4 of Fig. 3 shows that at 50 μM Ca++ the reaction with denatured DNA is complete in 8 min, whereas the accelerated phase with native DNA at 100 μM Ca++ begins after about 25 min. It seemed warranted to utilize this observation for probing DNA conformation with the enzyme. Fig. 8 shows that the difference in the rate of hydrolysis of native and denatured DNA both at 100 μM Ca++ is striking. Mixing the two substrates in different proportions produces a series of intermediate curves.

When devising these experiments, two alternatives were considered. The first was that, in a 50:50 mixture, denatured
DNA would be preferentially and completely digested, after which native DNA would be digested in a three-phase reaction. The second alternative was that throughout the whole course of the reaction both substrates would be digested, but to different degrees. The results shown in Fig. 8 exclude the first alternative. In agreement with von Hippel and Felsenfeld (7), the enzyme seems to bind native DNA more strongly than it binds denatured DNA. The duration of the slow phase is directly proportional to the amount of native DNA in the reaction mixture.

From an experiment in which both forms of DNA were simultaneously offered to the enzyme, Wingert and von Hippel (9) concluded that the denatured form is preferentially hydrolyzed. The same is true in our experiments, except that at no time is the preference for the denatured form so great as to remove it from the mixture before any of the native form is hydrolyzed.

Fig. 9 illustrates an experiment in which the hydrolysis of native and denatured DNA is compared under conditions where the reaction rate of the first phase is the same for both substrates. This was accomplished by adjusting the Ca\(^{++}\) concentration of the reaction mixture to 10 \(\mu M\) for denatured DNA and to 100 \(\mu M\) for native DNA. The enzyme and substrate concentrations were identical in both experiments. The results show that the reaction rate for the second (fast) phase is similar in both cases, but the duration is much longer with native DNA. As a result, the end point with native DNA is much higher than with denatured DNA, and the transition from the second to the third phase is less sharp. Addition of 0.15 M NaCl abolishes this difference as well as the triphasic nature of the reaction.

From previous experiments (Figs. 2 and 3), it was concluded that the end point of the reaction is dependent on Ca\(^{++}\) concentration. From experiments of Fig. 8, it is obvious that substrate conformation has no effect on the end point. Therefore, the results of Fig. 9 should be interpreted as evidence suggesting that the end point of the reaction can be regulated by an appropriate adjustment of Ca\(^{++}\) concentration, regardless of the conformation of the starting substrate.

**DISCUSSION**

The reason that autoacceleration has been overlooked for many years is probably because the originally established (20) optimum for Ca\(^{++}\) concentration has been up to now rather rigorously followed. Autoacceleration can only be observed at Ca\(^{++}\) concentrations which are considerably suboptimal (Figs. 2 and 3).

Micrococcal nuclease shows autoacceleration with all three substrates: RNA, denatured DNA, and native DNA. This is interpreted as evidence suggesting that the best substrates are polynucleotide chains of length intermediate between nucleic acids and short oligonucleotides. Presumably, these intermediate length chains show a sufficient amount of “breathing spaces” (9, 17) so as to be easily accessible to the enzyme. Work now in progress in this laboratory is directed toward correlating length and composition of substrate with occurrence of autoacceleration.

The results presented here establish that calcium exerts its dominant effect on the first phase of the reaction (Fig. 7). The rate of the second (fast) phase is apparently at its optimal plateau throughout the wide range of convenient Ca\(^{++}\) concentrations. The results, however, indicate that either the duration of the second phase or the rate of the third phase (or both) are dependent on Ca\(^{++}\) concentration. A higher Ca\(^{++}\) concentration permits more complete hydrolysis, as judged by the apparent end point of the third phase of the reaction (Figs. 2, 3, and 9).

It is very unlikely that a significant amount of double-stranded material is present at this time of the reaction, regardless of the original conformation of the starting substrate. The introduction of 0.15 M NaCl into the medium abolishes the delineation between the three phases with the appearance of a hyperbolic reaction rate curve. If the effect of NaCl at low Ca\(^{++}\) concentration is judged by the early phase of the reaction, it would be considered stimulatory; if by the later part, it appears inhibitory (Figs. 2 and 3).

Whether RNA or DNA is a better substrate depends on which phase of the reaction is examined (Fig. 6). This shows how conditional any statement about the preference of a nuclease toward substrate must be, when one is dealing with high molecular weight substrates. For micrococcal nuclease, a number of factors, such as conformation, chain length, and ionic medium, seem to be influencing the rate and apparent end point of the reaction to a much greater extent than the nature of the sugar moiety. It seems that chain length is the most important factor of those so far considered. Quantitative evaluation of preference toward denatured as opposed to native DNA must be equally conditional.

**REFERENCES**

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