INACTIVATION OF TRANSFORMING H. INFLUENZAE DNA BY DNases

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ABSTRACT

The inactivating effect of four different endonucleases on transforming activity of Haemophilus influenzae DNA was investigated.

Results obtained by viscosimetry, analytical band centrifugation analysis in neutral and alkaline solvents and tests of residual transforming activities have demonstrated that the inactivation levels by different enzymes are only function of the total number of phosphodiester bond breaks introduced into the starting native DNA molecules.

1. INTRODUCTION

Inactivation of transforming DNA by enzymic, chemical and physical agents has been extremely useful for understanding the fundamental steps in bacterial transformation, namely DNA

uptake and DNA integration into the host genome (Zamenhof, Alexander and Leidy, 1953; Lerman and Tolmach, 1957; Goodgal, 1961; Stuy, 1961;Bodmer and Ganesan, 1964;Bodmer, 1966;Cato and Guild, 1968; Bernardi and Bach, 1968 ; Nicolaieff and Chevallier, 1970; Thorsett and Hutchinson, 1971).

Inactivation by DNases has the advantage over inactivation by physical and chemical agents of being due to a single cause, namely phosphodiester bond breakage. It may be wondered, however, whether the degree of inactivation by DNases is only related to the number of bonds broken,or if it shows some relationship with the mechanism of action of the enzyme(single or double breakage of native DNA), or with the nature of the ends formed (3' or 5' phosphate). Results of Bernardi and Bach (1968) ruled out the first possibility,but not the second one. In fact, these authors showed that at comparable levels of degradation of H.influenzae DNA, spleen acid DNase, a 3'-phosphate former, was much more inactivating than either pancreatic DNase or E. coli endonuclease I, two 5'-phosphate formers. Indirect estimates of the total number of bonds broken by spleen and pancreatic DNases suggested that the different inactivation was not due to a larger number of bonds broken by the spleen versus the pancreatic enzyme hinting at a possible role of the nature of the ends. The subsequent report by Laipis,Olivera and Ganesan(1969)that the ratio of total number of bonds broken to the number of double breaks introduced by pancreatic DNase had been grossly overestimated by previous authors suggested to us that the conclusions of Bernardi and Bach (1968), who had used such estimates, might be incorrect. We were therefore encouraged to re-examine the problem of the inactivation of transforming DNA by DNases. While more detailed data will be reported elsewhere,we present here results obtained by degrading H. influenzae transforming DNA by four different

nucleases (spleen acid DNase, pancreatic DNase, E. coli endonuclease I, and micrococcal nuclease) and by correlating the inactivation of biological activity with the total number of breaks and the number of double breaks introduced by each enzyme. The results obtained show that inactivation of transforming H. influenzae DNA by the DNases used is only related to the total number of breaks introduced by the enzymes, independently of the nature of the ends formed. This conclusion is in agreement with the original proposal of Bodmer (1966) for B. subtilis DNA inactivation and with the results of Thorsett and Hutchinson(1971) on the D. pneumoniae DNA.

2. RESULTS AND DISCUSSION

DNase-treated DNA's were subjected to band sedimentation analysis in neutral and alkaline conditions (Studier, 1965). The sedimentation data were used to calculate double-stranded and single-stranded molecular weights, using the relationships of Studier(1965). Molecular weights were used, in turn, to estimate the number of double breaks and single breaks per million daltons of native DNA (Charlesby, 1954).

(a) Correlation between inactivation and double-stranded molecular weight of degraded transforming DNA

The correlation between double-stranded molecular weights and biological activity of the novobiocin resistance marker is shown in Fig. 1. Two major conclusions can be drawn from these results: 1) The inactivation curves markedly increase in slope below a molecular weight of about 15×10^6. 2) Large

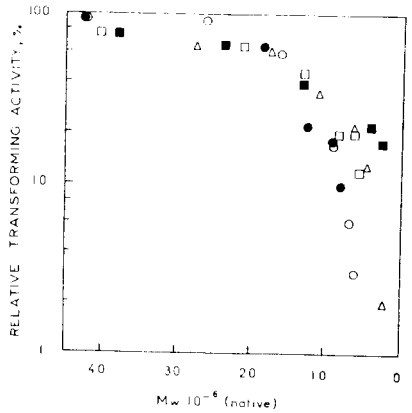

Fig. 1 - Inactivation of novobiocin marker of SN 668 DNA plotted against weight average molecular weights of samples treated by pancreatic DNase in presence of Mg^{++} (○), in presence of Mn^{++} (●), by spleen acid DNase (□), by E.coli endonuclease I(△)and by micrococcal nuclease(■). The residual transforming activities are given relative to that of the stock solution of SN 668 DNA, whose molecular weight was 55 million daltons.The enzyme treatments were performed on DNA dialyzed for 20 hours against the appropriate buffer : 0.15 M sodium acetate buffer-0.01 M EDTA, pH 5.0 for spleen acid DNase;0.02 M Tris-HCl buffer, 0.005 M $MgCl_2$, pH 7.5 for E. coli endonuclease I; 0.1 M borate buffer, $CaCl_2$ - 0.0025 M, pH 8.8 for micrococcal nuclease;0.05 M Tris-HCl, pH 7.5, supplemented with $MgCl_2$ (0.005 M)or $MnCl_2$ (0.00125 M),respectively,for pancreatic DNase.The transformation procedure used was that described by Goodgal and Herriott(1961).DNA concentration in transformation mixture was usually 1 µg/ml.

differences exist in the plots of residual transforming activity versus double-stranded molecular weight,as obtained by using different enzymes. On this basis, micrococcal nuclease is the least inactivating enzyme, followed by E. coli endonuclease I, spleen acid DNase and pancreatic DNase.

The differences in the inactivation curves shown in Fig. 1 are not related to the nature of the ends formed by different enzymes (3' or 5' phosphate) since 1) dephosphorylation of the 3' ends formed by spleen DNase and of the 5' ends formed by E. coli endonuclease I does not lead to any change in the residual transforming activity (Fig. 2), and 2) inactivation by spleen DNase and micrococcal nuclease, which are both 3'-phosphate formers, is very different.

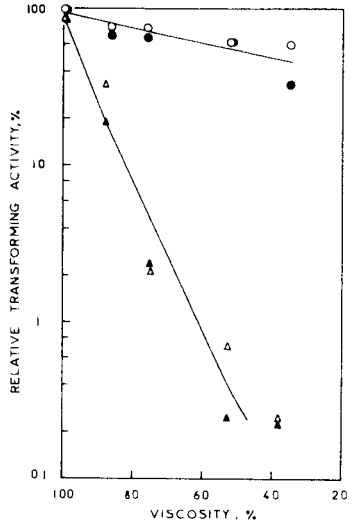

Fig. 2 - Inactivation of novobiocin marker in P^{32} labelled DNA of H.influenzae treated with spleen acid DNase; before dephosphorylation (△) and after dephosphorylation (▲). Inactivation of novobiocin marker in $EVSN_2B$ DNA degraded with E. coli endonuclease I; before (○) and after dephosphorylation (●).Dephosphorylation was carried out according to Weiss, Live and Richardson (1968).

In addition, differences in molecular weight distribution of DNA degraded by different enzymes are not responsible for the differences observed in Fig. 1, as shown by experiments

in which DNA samples degraded to comparable double-stranded molecular weights by spleen DNase and micrococcal nuclease were sedimented together in sucrose gradients (Fig. 3). The difference in specific activity in the fragments obtained after digestion with the two enzymes was practically the same for all fragment sizes; as expected, the largest fragments showed the highest specific activity.

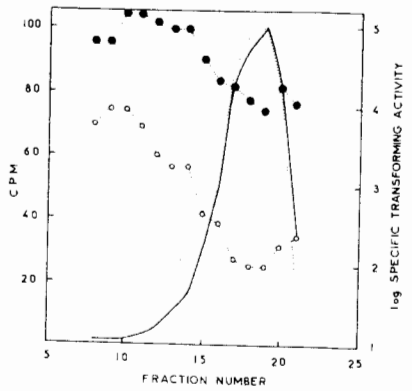

Fig. 3 - Sucrose gradient sedimentation of spleen acid DNase and micrococcal nuclease treated ^{32}P-DNA. ^{32}P-DNA samples degraded by spleen acid DNase and by micrococcal nuclease to a comparable viscosity (50%) were layered on sucrose gradient (4 ml, 5-20% in SSC) and sedimented at 38,000rev./min. for 2.5 hours at 10° C. 21 fractions were collected and analyzed for radioactivity (..... micrococcal nuclease treated sample) (—— spleen acid DNase treated sample), and for biological activity at non saturating DNA concentration (0,01 µg/ml). The biological activity is expressed as the log of specific transforming activity (i.e. log of number of transformants per 10^{-2} µg of DNA). ●.....● micrococcal nuclease treated DNA. O.....O spleen acid DNase treated DNA.

(b) Correlation between inactivation and single-stranded molecular weight of degraded transforming DNA

When residual transforming activity is plotted against the single-stranded molecular weight of H.influenzae DNA degraded by different enzymes, all data practically fit the same line (Fig. 4) showing the existence of a unique relationship between residual activity and single-stranded molecular weight of the fragments, regardless of the enzyme used.

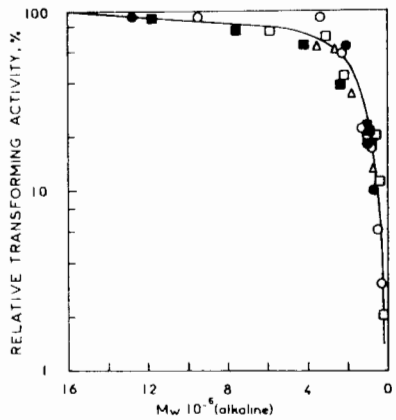

Fig. 4 - Inactivation of novobiocin marker of SN 668 DNA as a function of M_{20,w_A} determined in alcaline conditions. Results were obtained with DNA degraded by pancreatic DNase with Mg^{++} (○) and with Mn^{++} (●), by spleen acid DNase (□), by E. coli endonuclease I (△) and by micrococcal DNase (■).

The slope of the inactivation curve markedly increases below a single-stranded molecular weight of 3-4 x 10^6, a value significantly lower than half the value at which a similar phenomenon takes place for double-stranded molecular weight (Fig. 1). This phenomenon is explained by the presence of nicks in the

double-stranded DNA. Fig. 5 shows plots of residual transforming activity against the total number of breaks introduced by different enzymes per one million daltons of double-stranded molecular weight. As expected, points fall on the same line regardless of the enzymes used.

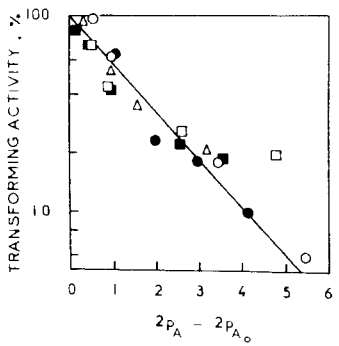

Fig. 5 - Inactivation of novobiocin marker plotted versus total number of breaks introduced by pancreatic DNase with Mg^{++} (○) and with Mn^{++} (●) as activators, by spleen acid DNase (□), by E. coli endonuclease I (△) and by micrococcal nuclease (■). $2pA - 2pA_0$ is the total number of breaks introduced by the enzymes per one million daltons of native DNA.

From the results presented in Figs.4 and 5,we can conclude that the level of residual biological activity in H. influenzae transformation, like in other transforming systems, (B. subtilis, Bodmer, 1966; and D. pneumoniae, Thorsett and Hutchinson, 1971) is a function of the size of single stranded DNA. This could be expected from the findings of Goodgal and Notani (1966)who demonstrated that in H. influenzae transformation only one strand of the DNA taken up by the recipient cells is incorporated into the host genome.

(c) Uptake of degraded DNA

Bernardi and Bach (1968) used competition experiments in order to show that uptake of transforming DNA was not differentially inhibited by spleen acid DNase and pancreatic DNase,and also that uptake was much less impaired than transforming activity,in agreement with Lerman and Tolmach (1957).We have confirmed this latter point directly in the case of spleen DNase degradation by measuring the uptake of tritium labelled DNA(Fig.6). In another experiment, penetration of spleen DNase-treated DNA before and after dephosphorylation was compared. DNA uptake and transforming activity were not modified by dephosphorylation. This result confirms the idea that 5' or 3' phosphate ends are not involved in inactivation,as already suggested by the previous results.

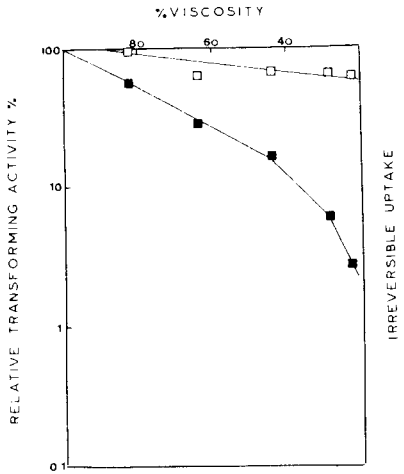

Fig. 6 - Decrease in transforming ability and uptake of ^3H-DNA treated by spleen acid DNase as a function of viscosity. Irreversible uptake was measured using the technique of Barnhardt and Herriott (1963).Transforming activity: ■——■ , irreversible uptake: □——□ .

3. REFERENCES

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