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Reversibility of Heat Denaturation of Monoalkylated DNA

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Induced partial reversibility of denaturation by treatment of DNA with methyl and ethyl methanesulfonate has been studied. At incubation of alkylated DNA at 37°C an increased reversibility appeared by time. When this DNA was incubated at a high temperature (60–70°C), where it was completely denatured, the reversibility disappeared. The time of appearance (at 37°C) and disappearance (at 60–70°C) was determined. The reversibility was found to be a function of the degree of alkylation independently of whether an ethyl or a methyl group had been introduced. The reversibility is probably caused by the aggregation of denatured DNA.

Reversibility (re-formation of the helix structure of DNA) of heat denatured DNA is induced after exposure of DNA to different agents, which cause cross-linking of DNA, *e.g.* difunctional alkylating agents,[1–3] nitrous acid,[4] ultraviolet light,[5,6] low pH.[7] Geiduschek has described two different kinds of reversibility.[2] Type I is a very rapid intramolecular process, which occurs in solutions of heterogeneous or homogeneous DNA at varying ionic strengths. Type II is a slow intermolecular process, which only can be studied with homogeneous DNA at moderately high ionic strength. The denaturation is graphically illustrated by plotting the increase of absorbance as a function of increasing temperature. The reversibility is measured as the absorbance at 25°C after quenching a denatured DNA solution and is illustrated by a curve similar to that of denaturation. It is possible to indentify "hidden" single-strand breaks. These breaks will inhibit the ability of the whole DNA molecule to reestablish its native structure after denaturation.

The monofunctional alkylating agents methyl, ethyl and isopropyl methane sulfonate (MMS, EMS and iPMS) show different mutagenic patterns.[9] In order to correlate these effects *in vivo* to chemical changes in DNA *in vitro* the denaturation and its type I reversibility were studied. Partial reversibility of DNA denaturation has been found after treatment with MMS and EMS.[8] Treatment of DNA with iPMS did not give this effect.[8] The present

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paper deals with factors influencing the appearance of the reversibility of heat denaturation of monoalkylated DNA. These factors are: time and temperature of incubation of treated DNA, and degree of alkylation.

MATERIALS AND METHODS

Calf thymus DNA (type I) (Sigma Chemical Co.) MMS and EMS (Eastman Organic Chemicals) were used.

DNA (0.75 mg/ml) was treated in a $NaHCO_3$ solution with the alkylating agent at 25°C. The solution was then chromatographed on Sephadex G-25 with 5×10^{-3} M citrate at pH 7. In some experiments the alkylated DNA was precipitated instead by 2 volumes of alcohol and washed.* The DNA solutions were concentrated to 1 mg/ml by evaporation in vacuum at room temperature. Samples of DNA were then incubated for different times at 37 or 50°C. The reversibility of the denaturation of alkylated and stored DNA was studied after complete heat denaturation and the disappearance of the reversibility was followed at 60, 65, and 70°C in either 50 % methanol or 7.2 M $NaClO_4$ at pH 7.

The DNA solution was heated step by step in 5°C intervals and kept at each temperature for 15 minutes and the transmission was registered. Then the samples were rapidly cooled to room temperature in an ice-bath and equilibrated to 25°C and the transmission was noted. The samples were alternatively heated and cooled. The study of the disappearance of the reversibility was made in a modified way. The sample of DNA was heated to 60, 65, or 70°C. Then the sample was kept at this temperature in a thermostat and the reversibility was measured after different times.

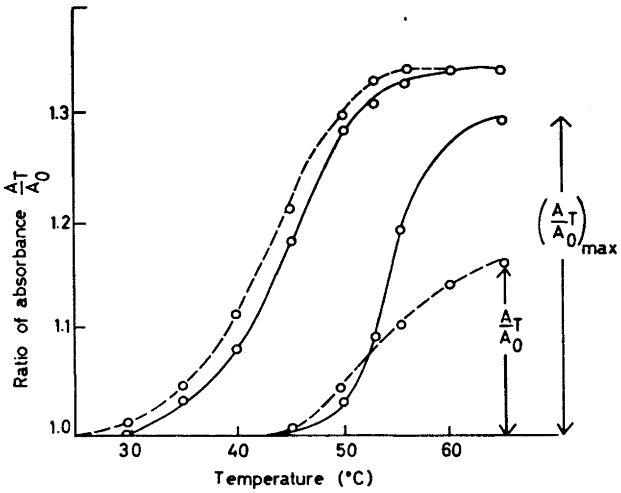

Fig. 1. Denaturation (the left pair) and reversibility (the right pair) curves of ethylated DNA immediately after treatment (solid line) and after incubation at 37°C (broken line). A_0 is the absorbance (at 254 nm) at 25°C (= 1) and A_T is the absorbance at the temperature T.

* When methylated DNA was chromatographed on a Sephadex column, optimal reversibility was always obtained even after very low degrees of alkylation. Precipitation of DNA by alcohol seemed to be a more efficient way to get rid of unreacted MMS. By using this procedure the degree of reversibility was shown to be a function of the degree of alkylation.

RESULTS

Fig. 1 illustrates the denaturation and its reversibility before and after incubation of alkylated DNA at 37°C. According to this figure the reversibility can be expressed as (if $A_0 = 1$)

$$(A_{T,\max} - A_T)/(A_{T,\max} - 1)$$

The reversibility of methyl and ethyl DNA is plotted as a function of time of incubation at 37°C (Fig. 2). The maximum reversibility of 60 – 70 % is obtained after about 60 hours at 37°C.

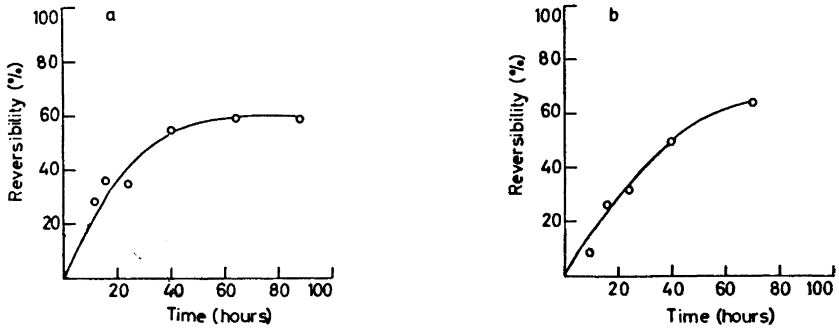

Fig. 2. Reversibility as a function of time of incubation of alkylated DNA at 37°C. a) Methylated DNA. b) Ethylated DNA.

By using the same terms as in Fig. 1 the decrease of the reversibility by time at incubation at a high temperature (60 – 70°C) is shown in Fig. 3. According to Fig. 3 the expression for calculation of the decrease of the reversibility can be written as

$$(A_{T,\max} - A_T)/(A_{T,\max} - A_{T,t=0})$$

Table 1. Rate constants for the disappearance of the reversibility after incubation of DNA at different temperatures. Treatment conditions of DNA by EMS: 0.4 M, 16 h and by MMS: 0.065 M, 75 min.

Alkyl-DNA	Temperature of incubation (°C)	Rate constant (mean ± st. error) (min^{-1})	Half-life (min)	Number of measurements
Ethyl-DNA	60	0.0038 ± 0.0007	182	3
	65	0.0069 ± 0.0005	100	4
	70	0.010 ± 0.002	69	4
Methyl-DNA	60	0.0033 ± 0.0004	210	3
	65	0.0070 ± 0.0006	100	6
	70	0.0098 ± 0.0016	71	4

The logarithmic values of the reversibility were plotted against time in a diagram and the reaction rate constant was calculated from the slope of the line. Table 1 shows the reaction rate constants and half-lives for the disappearance of the reversibility at different temperatures of incubation. As comparison, the disappearance of the reversibility of DNA denaturation obtained by treatment with the difunctional nitrogen mustard and succinate, respectively, at pH 3.75 was measured. The half-lives were 35 and 30 minutes respectively. The activation energy for the disappearance of the reversibility

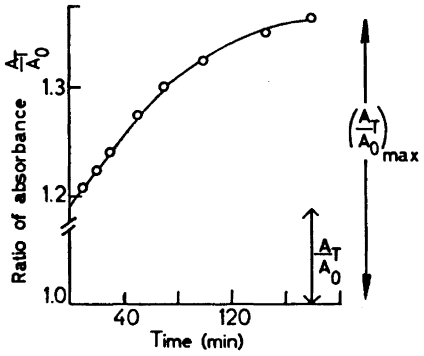

Fig. 3. Ratio of absorbance as a function of time of incubation at 70°C. Before the incubation the reversibility of the sample is $(A_T/A_0)_{t=0}$.

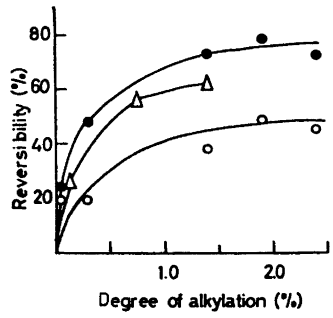

Fig. 4. Reversibility as a function of degree of alkylation (amount of alkyl groups/DNA-phosphate according to Walles and Ehrenberg[14]). The time of incubation at 37°C was for methylated DNA (△) 64 h and for ethylated DNA (○) 16 h and (●) 40 h, respectively. The treatment was for the different degrees of ethylation: 0.06 %: 0.015 M, 8 h; 0.3 %: 0.08 M, 8 h; 1.4 %: 0.40 M, 8 h; 1.9 %: 0.40 M, 12 h; 2.4 %: 0.40 M, 16 h, all at 25°C. The pH was kept about 7 by $NaHCO_3$. The treatment was with MMS at different degrees of alkylation 0.15 %: 0.03 M, 0.5 h; 0.72 %: 0.03 M, 2.5 h; 1.4 %: 0.06 M, 2.5 h at 25°C. The reaction mixture contained 0.065 M $NaHCO_3$.

of monoalkylated DNA was calculated. This was for ethylated DNA: 50 kcal, and for methylated DNA: 48 kcal. The maximum reversibility of complete heat denaturation of monoalkylated DNA is a function of the degree of alkylation (Fig. 4). The similar degree of ethylation and methylation gave about the same reversibility.*

* EMS treatment of DNA does not always induce reversibility. Under certain not yet identified conditions, e.g. concerning impurities in the DNA, no reversibility was obtained.

Table 2. Rate constants for the disappearance of the reversibility induced by different treatments of EMS.

Degree of alkylation (% per nucleotide)	Concentration of EMS (M)	Time of treatment at 25°C (h)	Rate constant at 65°C (mean ± st. error) (min^{-1})	Number of measurements
0.06	0.015	8	0.0060	1
0.3	0.08	8	0.0067 ± 0.0015	2
2.4	0.4	16	0.0069 ± 0.0005	4

DISCUSSION

The kind of reversibility, which was obtained after complete heat denaturation of DNA treated by MMS or EMS, was earlier referred to be due to cross-linking of the strands.[8] The origin of the cross-linking was supposed to be depurination as a secondary effect to alkylation. However, it has been shown [10] that treatment of DNA by iPMS leads to a rapid depurination. Isopropylation of DNA (0.9 – 3.7 %) [14] did not induce any increased reversibility.[8] Most probably the reversibility is caused by an aggregate formation of denatured DNA. It has been shown that methylation by MMS causes an increase of molecular weight of both DNA and poly A.[11–13] Lett [11] found also an increase of molecular weight after treatment of DNA by EMS. Ludlum [12] noted an increase of hypochromicity after alkali denaturation of methylpoly A. The increase in coiling of alkylated polymers was explained as a decrease of the net charge of the molecule at pH 7 as the pK_a values of the alkylated bases are much higher than those of non-alkylated. Then a slow transalkylation process should increase the amount of alkylated bases and thus the net charge should slowly decrease. However, it is obvious that the presence of alkylated bases is necessary for the appearance of this kind of reversibility. The bonds causing partially reversible monoalkylated DNA are of another kind than those induced by difunctional alkylating agents, which lead to complete reversibility. The breakage of those bonds at a high temperature has been referred to depurination.[3]

The appearance of a complete reversibility of the denaturation of monoalkylated DNA is counteracted by single-strand breaks and degradation. At incubation of alkylated DNA at a higher temperature (50°C) the highest obtainable reversibility for ethylated DNA was 50 % but at 37°C 70 %. This indicates an influence of single and/or double-strand breaks after EMS treatment.

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