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Minireview

Aggregated DNA in ethanol solution

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Abstract A recently developed mechanochemical method has provided a new, efficient tool for studies on the thermal stability and structure of aggregated DNA in ethanol–water solutions. At low ethanol concentrations DNA is fully soluble and is in the B form. However, with increasing ethanol concentration the melting temperature of DNA, T_m, decreases. At a critical ethanol concentration, dependent on the nature and concentration of the counterion, aggregation of the DNA molecules sets in. This is reflected in a marked increase in T_m indicating that the aggregated DNA molecules are thermally more stable than the dissolved ones. However, they are still in the B form. In general, T_m of aggregated DNA also decreases with further increasing ethanol concentration and is dependent on the nature of the counterion, but T_m is not affected by the concentration of the counterion (excess salt) in the ethanol–water solution. When the ethanol concentration reaches the range of 70–80% (v/v), the B-to-A conformational transition occurs in the case of Na-, K- and CsDNA. Above this transition point the A form is more stable than the B form due to the reduced water activity and to increased interhelical interactions. At very high ethanol concentrations, above 85% and dependent on the nature of the counterion, a drastic change in the thermal behaviour is observed. Apparently such a strong interhelical interaction is induced in the aggregated DNA that the DNA is stabilized and cannot adopt a random coil state even at very high temperatures. This stability of DNA in the P form is fully reversed if the ethanol concentration is lowered and the activity of water, thereby, is restored.

Key words: Nucleic acid; Secondary structure; Tertiary structure; Denaturation; Ethanol; Aggregation

1. Introduction

Nucleic acids are chemically linked sequences of subunits, each containing one of several nitrogenous bases, a pentose sugar and a phosphate group. The sequence of subunits is the form in which genetic information is stored in living organisms. While RNA is usually single stranded, a native DNA molecule consists of two complementary polynucleotide chains associated in a double helix. The sugar-phosphate residues are on the outside of the helix and carry negative charges on the phosphate groups [1]. In aqueous solutions these charges are neutralized by the occurrence of counterions. The bases lie on the inside of the helix, and they represent the hydrophobic part. The two polynucleotide strands are joined by secondary valence interactions between nitrogeneous bases: (i) hydrogen bonds between complementary bases as postulated by Watson and Crick [1], and (ii) interactions involved in base stacking (for review see ref. [2]). Molecular studies of the structure and thermodynamics of nucleic acids are essential for understanding the biochemical aspects of DNA in vitro and the biological properties of DNA in vivo. The task of this article is to review the current knowledge of the behaviour of DNA in ethanol-water solutions, particullarly at ethanol concentrations which cause aggregation of the DNA, whereby the function of hydration can be investigated.

2. DNA secondary structure

The helical structure of DNA can adopt several forms. The conformation is determined by, among other parameters, the activity of water, and by the nature of the counterions. In aqueous solutions, or in solid DNA at high relative humidities, the DNA helix occurs in the B form which has ten bases per turn and measures 34 Å per turn in the axial direction [3]. At moderate relative humidities a structural transition occurs, and NaDNA adopts the A form. This form is highly condensed and stabilized by interhelical interactions [4,5,6]. When the water activity is even lower, the C form may occur. The C form is closely related to the B form, but is less condensed and less crystalline [3] and it occurs also during premelting of DNA [2]. Some other forms of secondary structure, usually rare and dependent on very special conditions, have also been reported [7,8]. In general, when physico-chemical conditions change, various transitions can take place. The process of interconversion of DNA secondary structures occurs rapidly and is highly reversible. On the other hand, much less is known about tertiary and quaternary structures of DNA. When certain organic compounds, like polyethylene glycol, are added to dissolved DNA, various compact tertiary structures are induced [9].

3. DNA denaturation

The helical structure can be destroyed by very mild actions without requiring breakage of covalent bonds. For example, when DNA in aqueous solution is heated above the melting temperature, T_m, the interbase hydrogen bonds are broken and the two strands of the double helix separate and uncoil (for review see ref. [2]). The denatured state is maintained upon rapid cooling, and only some non-specific inter- and intramolecular base pairing occurs. However, if the thermally denatured DNA is cooled slowly, the process of reunion of the separated strands takes place and the original helical structure is largely restored [10]. A helix-to-coil transition may be more

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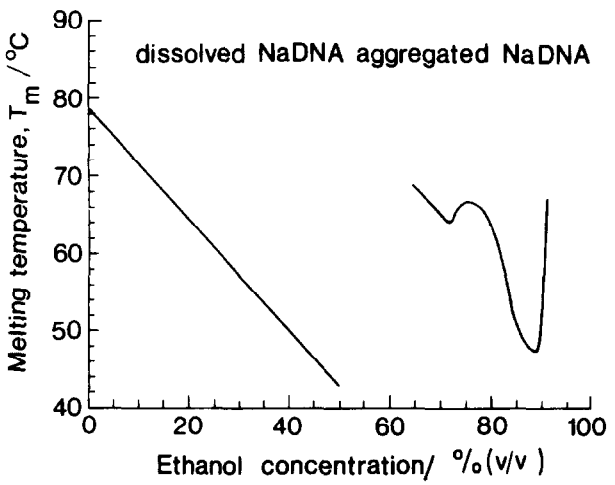

Fig. 1. The effect of ethanol concentration on the melting temperature, T_m, of NaDNA. The data for dissolved DNA are from ref. [14], and the data for aggregated DNA are from refs. [27,28]. More detailed information about the thermal behaviour of aggregated NaDNA is given in Figs. 2 and 4.

or less cooperative, depending on the particular solvent and DNA-counterion interactions [2]. In the ideal fully cooperative case all molecules are either in the helical or random coil state and the temperature width of the helix-to-coil transition is quite sharp. Two very important factors influencing the melting properties of DNA in aqueous solutions are the nature of the counterion and its concentration. The main role of the cation is to neutralize the electrostatic repulsion among negatively charged phosphate groups in DNA. The counterion stabilization of DNA to thermal denaturation decreases in the order: $Mg^{2+} > Li^+ > Cs^+, Na^+, K^+$ [11]. It appears that in aqueous solution there is a direct relationship between the Stokes radius of the hydrated counter ion and the T_m of DNA [12]. In other words, more hydrated counterions stabilize DNA better than less hydrated ones. At low salt concentrations, T_m is a linear function of the negative logarithm of the counterion concentration. This demonstrates the so-called salt effect on the thermal stability of DNA [2].

4. Dissolved DNA at low ethanol concentrations

The presence of alcohols in the solution has a large effect on the conformational and thermodynamic properties of biological macromolecules. When ethanol is added at low ethanol concentrations, NaDNA still remains in the B form, but T_m of DNA decreases [13,14] (Fig. 1). As the ethanol concentration increases and approaches 50% (v/v) there is a considerable tendency toward association of DNA molecules. Special precautions must be taken if DNA is to be studied in dissolved form up to 75% concentration [15]. At higher ethanol concentrations several conformational transitions, like B-to-A [16,17,18] and B-to-C [19] were observed for such dissolved DNA. Until recently studies of the heat-induced helix-to-coil transition at higher ethanol concentrations have been performed only on dissolved DNA. T_m was reported to decrease with increasing ethanol concentration, and a strong salt effect was observed [20,21]. In other words, the reduced activity of water in the ethanol solutions tends to destabilize the helical structure of dissolved DNA. However, as will be detailed below, the situation is drastically different with aggregated DNA.

5. Use of highly oriented fibers

Ethanol precipitation of DNA has been performed daily in practically every molecular biology laboratory. However, the properties of precipitated DNA at high ethanol concentrations have not been adequately understood [22]. The main reason for this has been the lack of satisfactory methodology for studies of aggregated DNA. However, mechanochemical study of highly oriented DNA fibers has recently provided useful information.

When the ethanol in aqueous ethanol solutions reaches about 50% (v/v), aggregation and subsequently precipitation of DNA occurs. The repulsive forces due to negatively charged phosphate groups are so diminished at these ethanol concentrations that interhelical contacts become possible. The induction of aggregation of DNA by ethanol, as well as the stability of aggregated DNA are greatly influenced by the nature and concentration of the counterion.

A wet spinning method has been used for over three decades for preparing samples of highly oriented DNA suitable for various physico-chemical studies [23,24,25]. This method was early modified for the preparation of a long fiber bundle of oriented DNA molecules from which a large number of reproducible samples could be taken for mechanochemical study [26]. Recently, a simple set-up was suggested for mechanochemical studies of conformational and helix-to-coil transitions in such DNA fiber samples [27,28,29,30]. A DNA fiber was held straight by a small weight in a measuring cylinder containing ethanol-water solution. The relative length of the fiber was

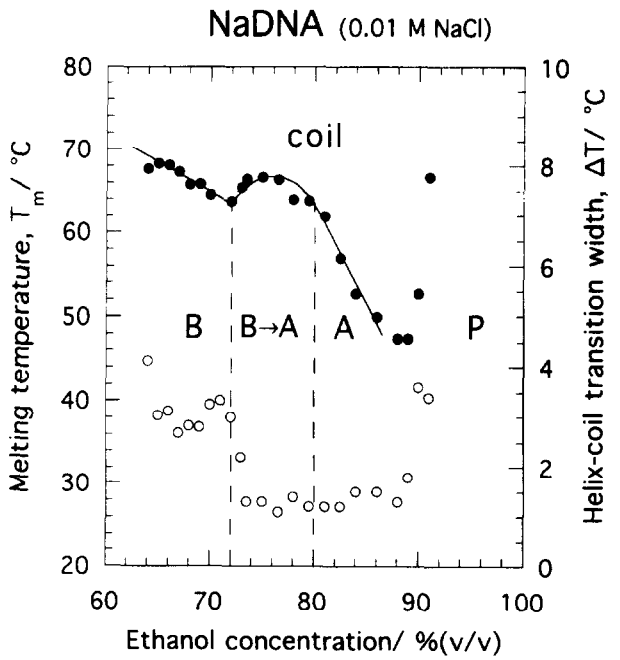

Fig. 2. Details of the thermal bahaviour of aggregated NaDNA. Melting temperature T_m (full circles), and transition width ΔT (open circles) for calf-thymus NaDNA fibers in ethanol-water solutions containing 0.01 M NaCl as a function of ethanol concentration. A, B and P refer to A-DNA, B-DNA and P-DNA, respectively. Note the local maximum in T_m in the B-to-A transition region. The data are from ref. [28].

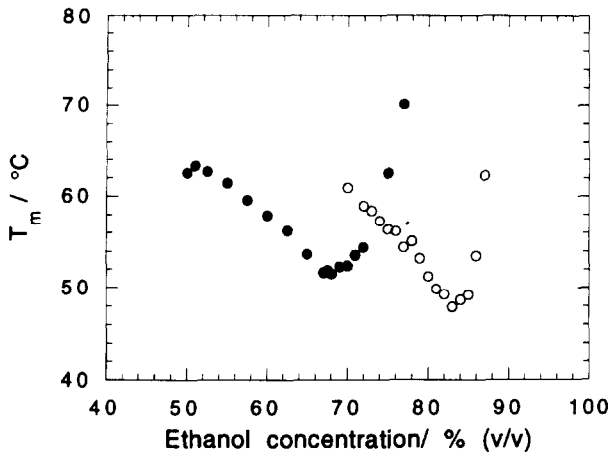

Fig. 3. The effect of ethanol concentration on the melting temperature of aggregated MgDNA (closed circles) and aggregated LiDNA (open circles). Note the difference between Mg- and LiDNA: (i) at lower ethanol concentrations MgDNA is more stable against disaggregation; (ii) the minimal value of T^m and (iii) the thermostable DNA structure are reached at much lower ethanol concentrations in the case of MgDNA. The data are from ref. [29].

recorded at different ethanol and salt concentrations and as a function of temperature. The main advantage of this system is that macroscopic changes of the fiber directly reflect microscopic events such as transitions of the secondary and tertiary structure of DNA. For example, the B-to-A transition of the double helix was recorded as a slight and reversible shortening of the fiber. These observations agree with results from X-ray diffraction [31,32]. A drastic and sudden contraction of the fiber upon elevating the temperature reflected the heat-induced helix-to-coil transition. This transition was not reversible. Such experiments were performed in different ethanol solutions containing various counterions, and in the following sections a simple model describing the behaviour of aggregated DNA in ethanol will be considered.

6. Conformational transitions and stability of DNA aggregates

The mechanochemical studies showed that Na-, K- and CsDNA underwent B-to-A transition, while LiDNA was suggested to undergo B-to-C transition when exposed to higher ethanol concentrations [28]. In the case of MgDNA, such transitions were not observed. The B-to-A transition was centered at 76% ethanol in the case of NaDNA, at 80% for KDNA and at 84% for CsCl; and the B-to-C transition was centered at 80% ethanol for LiDNA [28]. The A form is believed to be more firmly stabilized by aggregation and interhelical bonds. Li^+ and Mg^{2+} ions have the strongest hydration and subsequently the largest relative size, and therefore are likely to prevent the formation of the A form.

Mg^{2+} ions are more stabilizing for DNA aggregates than monovalent ions. Aggregated MgDNA, when reexposed to higher water activities, did not dissolve; instead gelation occurred [29]. Apparently, Mg^{2+} ions cause very strong aggregation of the DNA helices, an effect due to the high charge of the ion and the resulting increased importance of electrostatic interactions, particularly at high ethanol concentrations [29]. In the case of monovalent ions, increasing hydration destabilized aggregated DNA. The fibers began to dissolve in the order: Li- > K-, Cs- > NaDNA. In the case of aggregated LiDNA disaggregation began when the ethanol concentration was lowered below 70%, as concluded from the reduction of the fiber strength. Ethanol concentrations lower than 65% in the presence of K^+ and Cs^+ and below 60% for Na^+ were required for the corresponding effect in DNA fibers with these counterions [28]. These observations can explain the empirical practice in molecular biology laboratories [33]. Li^+ ions are added to avoid the precipitation of DNA at moderate ethanol concentrations, as in the case of differential RNA/DNA precipitation. Mg^{2+} ions are tied up during precipitation by adding chelating agents.

Table 1
Structure and melting behaviour of NaDNA in ethanol solutions

Ethanol concentration (v/v)	Structural transitions and T_m of DNA
0–50%	soluble DNA, B form, T_m decreases with increasing ethanol concentration, salt effect
50–60%	precipitation/aggregation occurs, interhelical interactions, B form, T_m drastically increased
60–72%	aggregated DNA, B form, T_m decreases with increasing ethanol concentration, low cooperativity, no salt effect
72–80%	aggreated DNA, B-to-A transition, further interhelical contacts, local maximum in T_m, higher cooperativity
80–92%	aggregated DNA, A form, T_m decreases with increasing ethanol concentration, higher cooperativity
above 92%	strongly aggregated DNA, P form, base stacking lost, thermally stable structure

The data are from refs. [27] and [28].

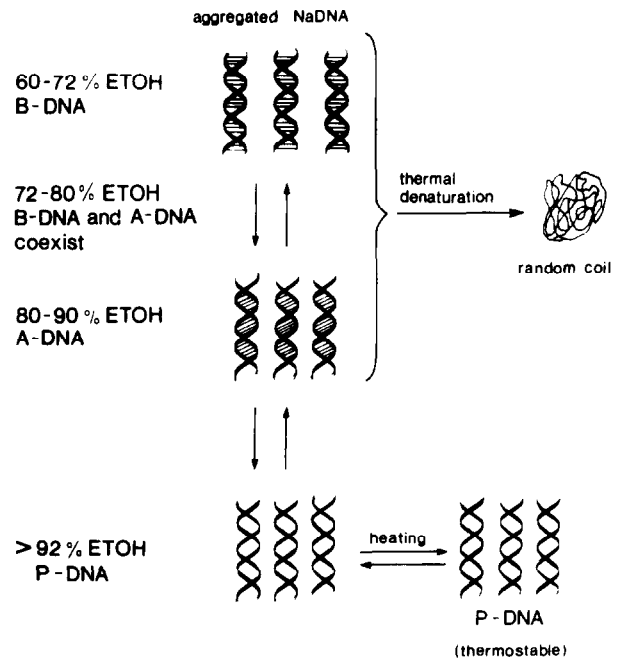

Fig. 4. A simple model describing thermal behaviour of aggregated NaDNA for various ethanol concentrations. Upon thermal denaturation of B-DNA and A-DNA the helix-to-coil transition takes place. In the B-to-A transition region, 72–80% ethanol (EtOH), B-DNA and A-DNA coexist. When transferred to >92% ethanol, NaDNA adopts the P form [37] which has altered secondary structure. Because of the very strong aggregation, P-DNA cannot undergo the helix-to-coil transition and is stable even at very high temperatures.

This inhibits nucleases and facilitates resuspension/resolubilization of ethanol-precipitated DNA pellets.

7. Thermal behaviour of aggregated DNA

The most interesting data resulting from the studies of DNA fibers were on the thermal behaviour of aggregated DNA in ethanol solutions. Previous to the employment of the mechanochemical method only the melting of dissolved DNA had been investigated [19,20,34]. At low ethanol concentrations, T_m decreases linearly with increasing ethanol concentration and reaches approximately 40°C at 55% ethanol in the case of NaDNA. This ethanol concentration is just below the value at which aggregation is induced [14] (Fig. 1). Similar results were obtained also for other counterions [35]. These observations were frequently extrapolated by molecular biologists who concluded that the DNA helix should be very unstable at room temperature when exposed to high ethanol concentrations.

The two significant parameters obtained from denaturation curves of DNA: the melting temperature, T_m, and the transition width, ΔT, could also be extracted from the mechanochemical melting curves of aggregated DNA. The last parameter, ΔT, reflects the cooperativity effect of the helix-to-coil transition and is defined as the mean-square deviation from the mean temperature. As illustrated for NaDNA in Fig. 1, T_m of aggregated DNA is much higher than T_m of dissolved DNA in the transition interval, 55–65% ethanol. DNA obviously becomes much more stable thermally when interhelical interactions occur. This is not due to a change of the secondary structure because the DNA is still in the B form.

The B-to-A transition influences the transition width, ΔT, of the helix-to-coil transition as well as T_m. When DNA adopts the A form, ΔT becomes narrower (Fig. 2) indicating increased cooperativity. Furthermore, there is a local maximum in T_m in the B-to-A transition region (Figs. 1 and 2, Table 1). Both parameters imply that the interhelical interactions for aggregated DNA drastically increase when the B-to-A transition takes place. This is in agreement with other studies suggesting that intermolecular bonds are important for the existence of the A conformation [36]. Aggregated KDNA behaves similarly while aggregated CsDNA lacks the local maximum in T_m [28]. After the A-to-B transition region follows a region with the DNA in the A form ending in a minimum in T_m (Figs. 1 and 2) at about 90% ethanol for aggregated Na-, K- and CsDNA [28].

In the case of aggregated Li- and MgDNA the changes are more gradual, because no B-to-A transition occurs, and T_m decreases with increasing ethanol concentration (Table 2). However, Mg^{2+} ions destabilize aggregated DNA much more than Li^+ (Fig. 3). The minimal value of T_m is reached at about 70% ethanol for aggregated MgDNA [29] (Fig. 3) and at about 84% ethanol for aggregated LiDNA [28] (Fig. 3). In both cases the minimum T_m is around 50°C. At even higher concentrations of ethanol a drastic change in thermal behaviour was recorded for all counterions (Figs. 2 and 3) as discussed in the next section.

Table 2
Structure and thermal stability of MgDNA in ethanol solutions

Ethanol concentration (v/v)	Structural transitions and T_m of DNA
0–40%	soluble DNA*, B form
40–50%	precipitation/aggregation, B form
50–70%	aggregated DNA, B form, T_m decreases with increasing ethanol concentration
above 70%	aggregated DNA, P form, thermally stable structure

The data are from ref. [29].
*Once MgDNA has aggregated, it does not redissolve easily, even when the ethanol concentration again is lowered, i.e. after restoring very high activities of water.

In general T_m is a function of ethanol concentration as well as the nature of the counterion. The ions stabilize aggregated DNA in the following order: $Mg^{2+} < Li^+ < K^+$, Cs^+, Na^+. Therefore, it seems that the thermal stability of DNA in ethanol varies inversely with the size of the solvated counterion which is just the opposite of the results obtained in solutions lacking ethanol. Furthermore, while the melting of dissolved DNA in water is strongly influenced by the salt concentration, no salt effect on T_m is observed for aggregated DNA [28] (Table 1), possibly with the exception of LiDNA at very low salt concentrations [28]. One explanation is that the salt dependence of the electrostatic contribution to the free energy difference between coil and helical conformation(s) is negligible in the highly packed fiber system. However, it should be stressed that the salt concentration plays an important role in the induction of DNA aggregation and subsequent precipitation. Therefore, addition of excess salt is recommended for efficient ethanol precipitation [22,33].

8. Aggregated DNA at very high ethanol concentration

Higher ethanol concentrations than those found to give minimum values of T_m of aggregated DNA begin to thermally stabilize aggregated DNA in the presence or absence of excess salt. As a result, T_m increases rapidly (Figs. 1, 2 and 3). However, the increase in the transition width at these ethanol concentrations (Fig. 2) is probably a kinetic effect [28]. At a certain ethanol concentration, DNA does not melt at all, not even when the ethanol solution has reached the boiling point (Fig. 4). The ability to form a thermostable DNA structure at high ethanol concentrations is influenced by the nature of the counterion and decreases in the order: $Mg^{2+} > Li^+ > Na^+ > K^+ > Cs^+$ [28,29]. This behaviour may be explained as follows: before the critical concentration of ethanol is reached the aggregation forces are only moderately strong and single strands of the aggregated DNA can slip past each other in the endeavour to form a random coil upon thermal treatment, yielding fiber contraction. However, above the critical ethanol concentration the interhelical interactions have become so strong that the tertiary structure is stabilized and DNA can not adopt a random coil structure. As a result of this the fiber will not contract. Thermal instability is fully restored when the ethanol concentration is lowered and the activity of water again is increased (Fig. 4). Crystallography studies on DNA exposed to high ethanol concentrations indicated that base stacking had disappeared, while the helical structure and molecular orientation remained [28] (Fig. 4). These results are analogous to the behaviour of the P form of DNA observed in solvent mixtures of high methanol and methanol-ethanol concentrations [37] and the behaviour of the soluble fraction of DNA at high ethanol concentrations [19]. The P form is thought to have altered second-

ary structure with the almost complete absence of hydrogen bonding and base stacking, while the tertiary structure is condensed (Fig. 4). A similar description could be valid also for dry DNA, which presumably lacks base stacking, but the native structure can be reformed on rehydration [38].

In conclusion, aggregated DNA seems to be thermally relatively stable at any ethanol concentration. Probably, stability is conferred by interhelical interactions in the aggregates. Previously, it has been demonstrated that lower temperatures do not facilitate aggregation and precipitation [22]. It may be concluded that ethanol precipitation of DNA, as performed routinely in every molecular biology laboratory does not require low temperatures either for increased stability or aggregation/precipitation.

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