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Characterization of Imidazole as a DNA Denaturant by Using TGGE of PCR Products from a Random Pool of DNA[1]

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Perpendicular temperature gradient gel electrophoresis (TGGE) profiles were analyzed for PCR products from a random pool of DNA [60 nts random region flanked by two primer (20 nts) sites]. Besides a normal transition profile of a homoduplex, unique mobility transition profiles of two kinds of heteroduplex with a big internal loop were observed, representing the successive helix-coil transitions of the DNAs. As the appearance of the heteroduplex band is an estimator of the complexity of a random pool, it will be applicable to monitor the extent of the selection process in the *in vitro* selection method. When imidazole was added to the electrophoretic buffer, the transition pattern shifted to the low temperature side. At a concentration of 1 M, imidazole lowered the melting temperature (T_m) of DNA by 13±2°C for all the three chain separation transitions observed. Thus imidazole is a stronger denaturant than urea, at least at dilute concentration. Dependence of T_m on concentration of imidazole and the mobility change suggested that imidazole binds to nucleotide in the single-stranded state.

Key words: imidazole as a DNA denaturant, *in vitro* selection, ligand-induced melting, melting temperature of DNA, TGGE.

In evolutionary molecular engineering (*1, 2*) PCR is often performed for a random pool of short DNAs (*3*). Even if the final cycle is finished at the elongation step, there exists a heteroduplex hybridized at primer-binding regions at the both ends and with a big internal loop corresponding to a random sequence region. The fraction of the heteroduplex increases as PCR cycle number increases and the primer concentration decreases. The hybridization to the perfectly complementary strand is too slow to form a homoduplex during the PCR annealing step, whereas formation of the heteroduplex can take place when the primer concentration is low. This situation is similar to that of C_0t analysis for fragments of DNA from a eukaryote repetitive sequence (*4*).

Perpendicular temperature gradient gel electrophoresis (TGGE) (*5*) has been used to study the melting characteristics of a mixture of DNAs (*6, 7*). The electric field is applied perpendicular to temperature gradient. In this article we report that the perpendicular TGGE of the PCR products from a random pool of DNA gave unique profiles of the mobility transitions associated with the helix-coil transitions of the heteroduplex DNAs. As these transitions were clearly displayed on the gel plate, the effects of denaturant on the melting temperature (T_m) were easily measured in these experiments.

The effect of denaturants (urea or formamide) on DNA melting had been found experimentally to be equivalent to that of temperature, and the conversion factors were determined (*7, 8*).

In our study on evolutionary molecular engineering of single-stranded (ss) DNA, we discovered that imidazole eliminates PCR sub-bands (*9*). This suggests that imidazole can destabilize the double-stranded form of DNA just like urea and formamide. In this article, we report secondly that imidazole is a stronger denaturant of DNA than urea and suggest a plausible mechanism of destabilization. This result is based on above-mentioned experiments using TGGE.

MATERIALS AND METHODS

Reagents—Reagent grade materials were used throughout without further purification. PCR kit was obtained from Greiner Labortechnik. *N,N'*-Methylene-bisacrylamide, acrylamide, TEMED (*N,N,N',N'*-tetramethylethylenediamine), APS (ammonium persulfate), Tris (2-amino-2-hydroxymethyl-1,3-propanediol), EDTA (ethylenediamine-*N,N,N',N*-tetraacetic acid), sodium acetate, urea, and imidazole for gel and gel electrophoresis, and other common reagents such as silver nitrate, ammonia solution, and sodium hydroxide were from WAKO Pure Chemical Industries.

Preparation of DNA Sample for TGGE—Random single-stranded DNA pool (5′ GTCGGATCCTAGCTCCACAT $(N)_{60}$ TGGTTCGCTAAGCTTGAGCC 3′) of diversity 10^{10} (100 fmol) was from Oligo Service. It was amplified in 100 μl of PCR buffer [50 mM Tris-HCl, pH 8.8; 15 mM $(NH_4)_2$-

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Abbreviation: nts, nucleotides.

SO_4; 2.5 mM $MgCl_2$; 0.45% TritonX-100; 200 μg/ml BSA) including 10 pmol of each primer (**pp**:5′ GTCGGATCCTA-GCTCCACAT, **pm**: biotin-- 5′ GGCTCAAGCTTAGCGA-ACCA), 200 μM dNTPs, 1 unit Taq DNA polymerase. The mixture was cycled 30 times on a thermal cycler (MJ Research) after it had been treated at 95°C for 3 min. The temperature cycle was 94°C for 30 s, 66°C for 30 s, and 72°C for 30 s. The final cycle was finished after the elongation step, and the mixture was cooled at room temperature. A 20 μl portion of the PCR product was then mixed with loading dyes (0.3% Xylene cyanol ff, 0.3% phenol red, 20% saccharose).

Temperature Gradient Gel Electrophoresis (TGGE)—Six percent polyacrylamide (*N,N′*-methylenebisacrylamide:acrylamide = 1:19, 1 × SAE buffer) gels with various concentrations of imidazole or urea were prepared. The size of glass plate was 16 × 16 × 0.1 cm. The running buffer was 1 × SAE (40 mM Tris, 20 mM sodium acetate trihydrate, 1 mM EDTA, pH 8.0) unless otherwise noted. In the case of the gel with imidazole, the concentration of imidazole was same both in the gel and in the running buffer. The pH of the running buffer was measured after addition of imidazole. After the gel had been prerun for 15 min, the sample was applied directly as a continuous line across the top of the gel. The electrophoresis was performed at 9 V/cm for 10 min at room temperature to bring the sample into the vertical gel. The gel was then bonded to a sheet (Gelbond film 160 × 170 × 0.2 mm; FMC) and placed closely on the horizontal flat plate of a temperature gradient generator (GT-180/GC-80, TAITEC). The gel was incubated for 30 min with the power supply off in order to establish the desired temperature gradient (usually from *ca.* 30 to 80°C). Then the perpendicular TGGE was performed for 2 h at 29 V/cm (approximately 400 V, 40 mA). The temperature of both high and low temperature sides of the gel was monitored by use of a thermister thermometer (D613, Takara Thermister Instrum.).

Staining—After electrophoresis, the gels were soaked in distilled water to remove denaturant (imidazole or urea) and stained with silver.

RESULTS AND DISCUSSION

Figure 1 shows schematically the various DNA molecules in our electrophoretic gel matrix. When PCR of random DNA of great diversity is finished at elongation step, the PCR tube contains ds DNA, ss DNA, and ds DNA with a big internal loop. After 20 cycles, the concentration of primers becomes comparable with that of the already amplified template, and some fraction of the two templates hybridize with each other by recognizing the priming sites at both ends. As the inner region is of random sequences, it forms a big internal loop of loop size 120. The ds DNA with a big internal loop must take a shape something like a two rods (composed of 20 bp ds DNA) connected with a flexible joint. In the electric field in the gel matrix, this type DNA should sometimes take a U-shape, hanging on a gel fiber at the flexible joint. This hanging mechanism may lower the electrophoretic mobility in comparison with simple rod-like molecule (100 bp ds DNA). The broom-like molecule may have even lower mobility than the U-shaped molecule.

Electrophoretic patterns of PCR product on temperature gradient gel with various concentrations of imidazole are shown in Fig. 2. The direction of migration is from top to bottom and the temperature gradient increases from left to right. The overall linear increase in mobility of the DNA as temperature increases is due to the effect of temperature on viscosity and pore size of the gel.

TGGE Profile of PCR Products from a Random Pool of DNA—A schematic representation of the melting profile on the TGGE gel plate and its interpretation are shown in Fig. 3.

TGGE without any denaturant gave a profile showing the temperature-induced mobility transition associated with the conformational transition of duplex DNA (Fig. 2A). At the high temperature side, there are two bands of single-stranded (ss) DNA: a main band (100 nts) and a sub-band (*ca.* 200 nts). At low temperature, there are three bands: ⟨a⟩, ⟨b⟩, and ⟨d⟩. Band ⟨a⟩ corresponds to the mobility transition curve of the DNA that has a big internal loop clamped by a strong (**pp**) and a weak (**pm**) primer site. It has lower mobility than the random coil ssDNA . This DNA melts *via* two transitions. The first one is based on the dissociation of one of the two primer sites, and the conformation of the DNA changes from the clamped-at-both-sites state (an internal loop) to the clamped-at-one-side state. Transition temperature T_{m1} was 53°C.

Local melting temperature of a local cooperatively melting region is defined as the temperature at which the helix content is 1/2 of the region. As we can not identify the point of the helix content of 1/2 on the gel plate, we defined the apparent melting temperature (or transition temperature, or chain separation temperature) T_m as the half-way point of mobility change similarly to the previous study (7).

The second transition is based on the whole chain separation. The chain separation temperature T_{m2} was 58°C. Although the two priming regions are same in size and GC content, the total stacking free energy of these two regions is different. Thus the local melting temperature of these regions is different (T_{m1} and T_{m2}). A theoretical thermal melting process of the DNA with a big internal loop is shown in Fig. 4. The calculation was carried out based on the four-states model (internal-loop state, left-side-open

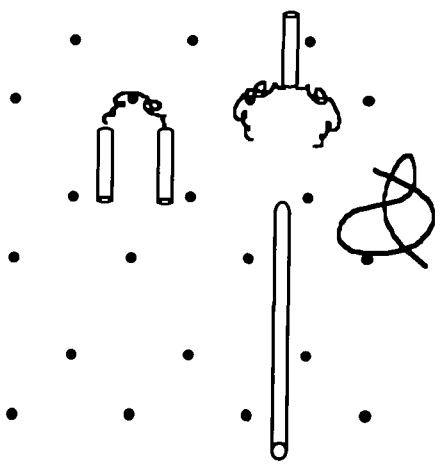

Fig. 1. **DNA in various conformations migrating under an electric field in the gel matrix.** The mobility of these conformations decreases in the orders rod-like molecule > random coil > U-shaped molecule > broom-like molecule.

state, right-side-open state, and two-random-coils state) using the nearest-neighbor stacking parameters (*10*), the loop entropy of loop size 120, and the concentration of the DNA strand in the gel estimated from that of the sample solution. We got qualitatively good agreement with experimental result. The first transition corresponding to T_{m1} in Fig. 3 is identified to be the dissociation of the **pm** region. Therefore, a nearly double size random coil of the slowest mobility is formed at the middle of the melting process. The theoretical value of $T_{m2} - T_{m1}$ is nearly twice as large as

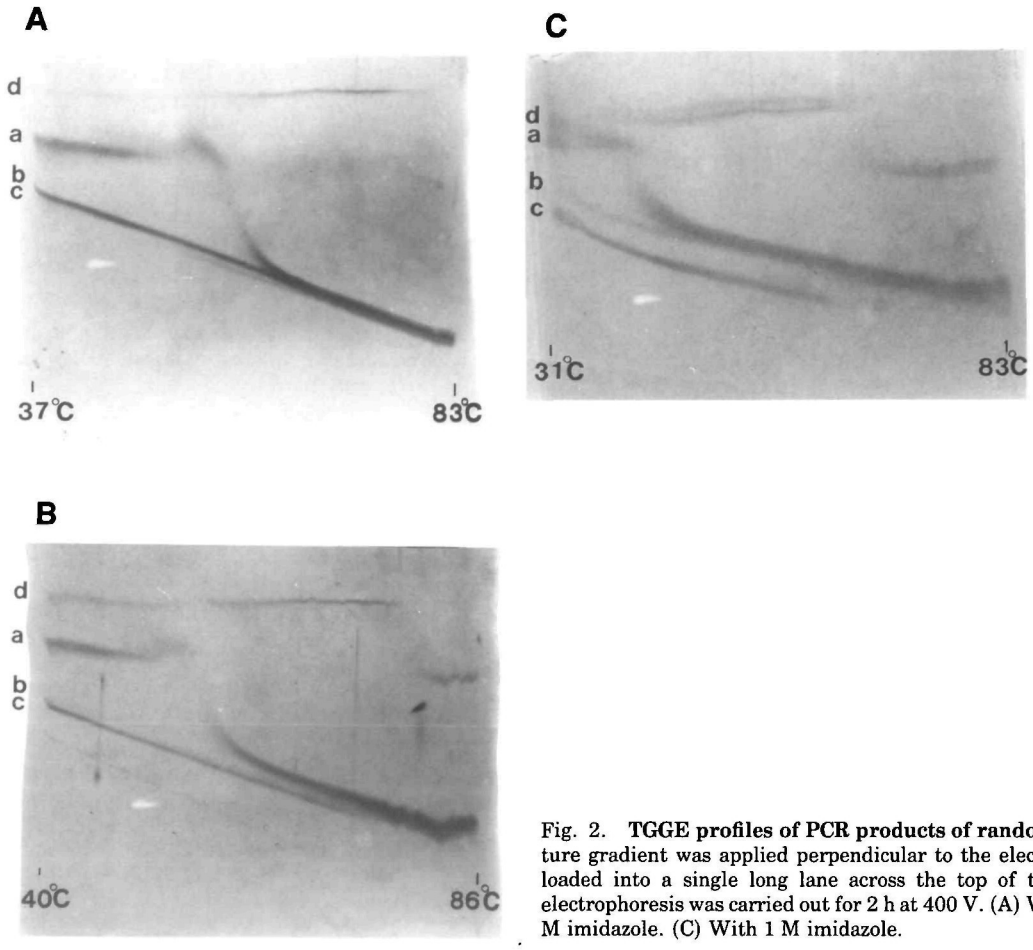

Fig. 2. **TGGE profiles of PCR products of random pool DNA.** A linear temperature gradient was applied perpendicular to the electric field. The DNA sample was loaded into a single long lane across the top of the 6% polyacrylamide gel, and electrophoresis was carried out for 2 h at 400 V. (A) Without denaturant. (B) With 0.1 M imidazole. (C) With 1 M imidazole.

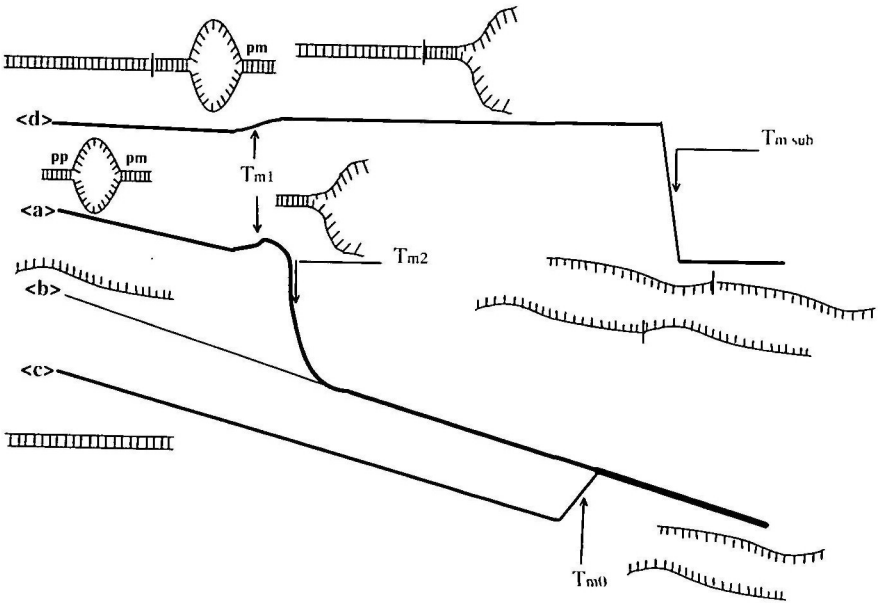

Fig. 3. **Schematic representation and interpretation of Fig. 2B.** Also shown are various apparent T_ms. The assignment of **pp** and **pm** is based on the data in Fig. 4.

the experimental value. Possible reasons are (i) the difference between true T_m and apparent T_m, especially for the chain separation step, and (ii) the effect of gel matrix on the loop entropy and on the activity of buffer and of DNA.

Because the internal loop is composed of random sequences, it may take diversified sub-structures. The TGGE band of this ds DNA at the transition region is broader than the perfect ds DNA (Fig. 2). One reasons for the broadness is probably these substructures, although the main reason must be the smaller effective ΔH for the duplex with a big internal loop.

Band ⟨b⟩ at the low temperature side in Fig. 2A is the superposition of two bands from ds DNA and ss DNA, which happen to have the same mobility. In other words, the viscous resistance in the ss state is nearly a half of that in the ds state. In this case, we could not measure the melting temperature for the perfectly matched ds DNA.

The slowly migrating sub-band ⟨d⟩ was identified as corresponding to the head-to-tail dimer of the original DNA template (*9*). PCR of the dimer generates a ds DNA with a big internal loop (size = 60 × 2 = 120 nts) flanked by a short stem (20 bp) and a long stem (120 bp) using an already amplified template monomer as a primer. At the temperature around T_{m1} or T_{m2}, the short stem melts to generate a conformation composed of the long stem (120 bp) plus an (80+80) nts coil region at the one end (a broom-like molecule). The electrophoretic mobility of this molecule is much lower than that of a fully separated coiled molecule (200 nts). Therefore at the temperature $T = T_{m\,sub}$ where the long stem melts, the band drops sharply as shown in Fig. 2. The chain separation temperature $T_{m\,sub}$ was 79°C. Curiously, the normalized mobility of the broom-like molecule gradually decreased as temperature increased; here the normalization is performed using the mobility of the standard dye molecule (*6*).

Just as in the C_0t analysis, the rate of renaturation (or hybridization) of the heteroduplex depends on the diversity of DNA sequences. Thus, the appearance and elimination of band ⟨a⟩ is an estimator of the complexity of the random pool of DNA. This type of TGGE is applicable especially to monitor the extent of the selection process in the *in vitro* selection method (data not shown).

Imidazole as a Denaturant—To evaluate the denaturing power of imidazole, we performed TGGE in which both the gel and running buffer contained 0.1, 0.3, 1.0, or 3.0 M imidazole. Results are shown in Fig. 2, B and C, for 0.1 and 1.0 M respectively. When the gel contained the denaturant, the mobility of the random coil was lower than that of the perfectly matched ds DNA, and the melting of the DNA (100 bp) was observed at temperature $T = T_{m0}$. It is evident that T_{m0}, T_{m1}, T_{m2}, and $T_{m\,sub}$, decreased with the increase in imidazole concentration. At a concentration of 1.0 M, imidazole lowered all four melting temperatures of short DNA by 13 ± 2°C, although the data of T_{m1} is not so precise as the others. The data are plotted in Fig. 5A. The same

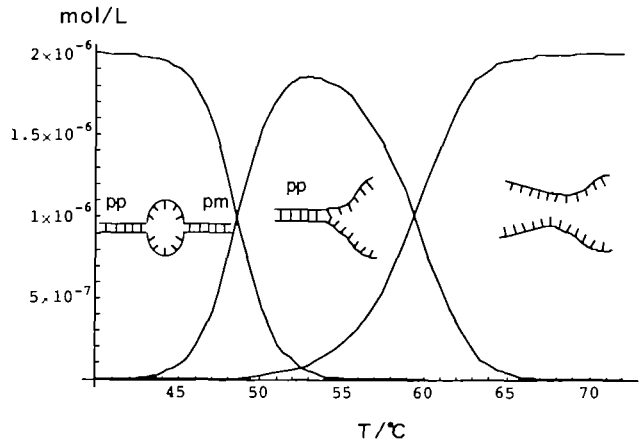

Fig. 4. **Theoretical melting process of the DNA duplex with a big internal loop based on the four-states model (see text).** The ordinate is the molar concentration of three molecular species, that is, ds with a big internal loop, ds clamped only at the **pp** site, and ss(+). The abscissa is the temperature (T). Mole fraction of ds clamped only at the **pm** site is calculated to be negligibly small. Thermodynamic parameters (ΔH and ΔS) used are for 0.19 M Na^+ (*11*).

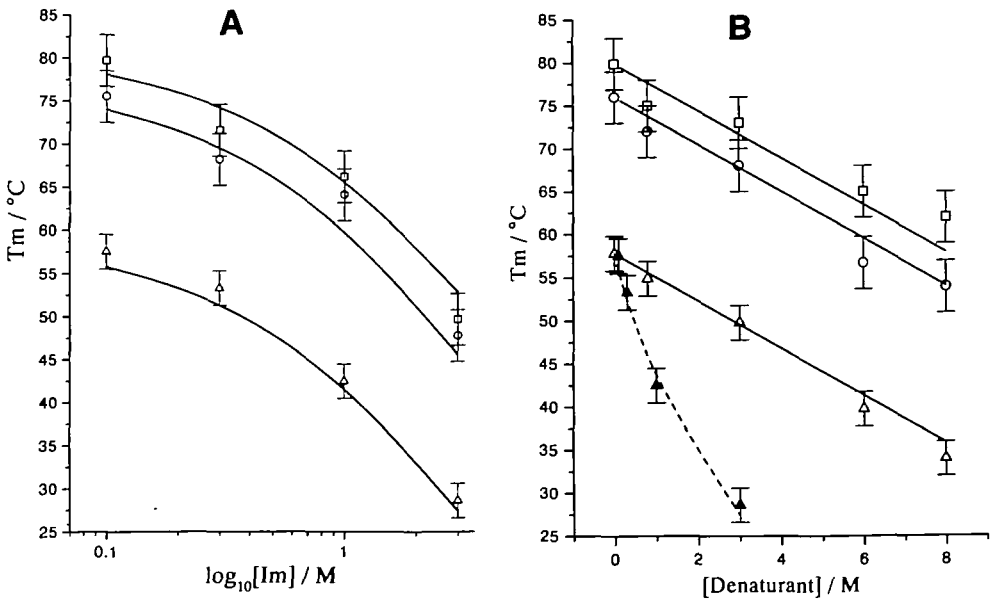

Fig. 5. **Effect of the concentration of imidazole (A) and urea (or imidazole) (B) on the melting temperature of DNA.** The ordinate is the melting temperature. The abscissa is the denaturant concentration, on a logarithmic scale (A) or a linear scale (B). □: Melting of sub-band, $T_{m\,sub}$. ○: Melting of perfectly matched ds DNA, T_{m0}. △: Chain separation of the ds DNA with a big internal loop, T_{m2}. Solid lines in (A) are theoretical curves (Eq. 2) fitted to the experimental data. $K^{(c)} = 0.739$ M^{-1}. Solid lines in (B) are $T_m(c) = T_m(0) - \alpha c$, where $\alpha = 2.71$ °C M^{-1} and c is the molar concentration of urea. Dashed curve (▲) in (B) is for T_{m2} in imidazole denaturation.

experiment was performed with urea (Fig. 5B) for comparison.

The mechanism of destabilization of DNA double helix by imidazole is postulated to be as follows. Imidazole binds more tightly to nucleotide in the ss state than in the ds state. Chemical thermodynamics of the ligand-induced melting of DNA in this model gives the decrease of the melting temperature T_m as follows:

$$1/T_m(L) - 1/T_m(0) = (R/\Delta H) \cdot \ln(1 + K^{(c)}L)/(1 + K^{(h)}L)), \quad (1)$$

where L, $K^{(c)}$, $K^{(h)}$, ΔH, and R denote the ligand concentration, the association constant to ss-state nucleotide, that to ds-state nucleotide, the transition enthalpy, and the gas constant, respectively. Here we assumed that the number of ligand-binding sites is the same for both states. The derivation of Eq. 1 is available from the authors upon request (see also Ref. *11*). In a simpler version of the model, imidazole binds only to nucleotides in the ss-state. As the range of T_m change is very narrow, the above equation is nearly equivalent to the following one in the simpler version:

$$T_m(L) = T_m(0) - (RT_m(0)^2/\Delta H) \cdot \ln(1 + K^{(c)}L). \quad (2)$$

The solid lines in Fig. 5A are the fitted curves expressing this equation, in which apparent T_m is taken as the true T_m. There is no linear relationship between the decrease of T_m and concentration of imidazole (dashed curve in Fig. 5B).

The change in true T_m deriving from the change in the denaturant concentration must be nearly equal to the change in apparent T_m, although the true T_m itself may be appreciably different from the apparent T_m. If the ligand (imidazole) binds cooperatively, the curve should have a plateau-followed-by-cliff shape (*11*) rather than this gradual convex type. Therefore, imidazole seems to bind non-cooperatively to the nucleotide in the ss state.

When the gel contained imidazole, the ss DNA was of lower mobility than the perfect ds DNA. As the concentration of imidazole increased, separation between the ss-state band and the ds-state band increased. This phenomenon is compatible with the hypothesis of binding of imidazole to the ss-state. As the same phenomenon was observed for urea, which has probably a different denaturation mechanism, as discussed below, this compatibility does not mean proof.

When the SAE buffer contained 1 M imidazole, the pH of the solution increased to 9.0. To clarify the effect of pH, we prepared the SAE buffer of pH 9.0, without imidazole. No difference was found in the TGGE profile and melting temperature from the data at pH 8.0 (data not shown).

As pK_a of imidazole is 6.5 at 50°C, almost all (99%) molecules are not charged at pH 8.5. Thus, imidazole cannot contribute to the stabilization of DNA double helix by a charge effect.

Imidazole hydrolyzes RNA by the general acid-base mechanism (*12*), just as histidine does. In contrast to RNA, no such effect is observed for DNA, because of the lack of a 2′ hydroxyl group. Imidazole, however, destabilizes the DNA double helix. Histidine was proved also to be a denaturant of DNA as measured by the elimination of PCR sub-bands (*9*). These phenomena are analogous to alkaline degradation of RNA and alkaline denaturation of DNA.

As seen in Fig. 5B, the destabilization effect of urea is different. A linear relationship between the decrease of T_m and concentration of urea is evident. Solid lines in Fig. 5B confirm the empirical relationship previously established (*7, 8*); 2.7°C decrease of T_m per 1 M increase of urea concentration. It is generally accepted that the denaturation mechanism with urea is the destruction of the liquid structure of water rather than ligand-induced denaturation.

As mentioned above, 1 M imidazole lowers the melting temperature of DNA by 13±2°C. In conclusion, imidazole is a much stronger denaturant of DNA than urea, at least at dilute concentration.

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REFERENCES

1. Eigen, M. and Gardiner, W. (1984) Evolutionary molecular engineering based on RNA replication. *Pure & Appl. Chem.* **563**, 967-978
2. Husimi, Y. (1989) Selection and evolution of bacteriophages in cellstat. *Adv. Biophys.* **25**, 1-43
3. Elington, A.D. and Szostak, J.D. (1990) *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818-822
4. Britten, R.J. and Kohne, D.E. (1968) Repeated sequences in DNA. *Science* **161**, 529-540
5. Riesner, D., Henco, K., and Steger, G. (1991) Temperature gradient gel electrophoresis: a method for the analysis of conformational transitions and mutations in nucleic acids and proteins. *Adv. Electrophoresis* **4**, 169-250
6. Nishigaki, K., Tsubota, M., Miura, T., and Husimi, Y. (1992) Structural analysis of nucleic acids by precise denaturing gradient gel electrophoresis: I. Methodology. *J. Biochem.* **111**, 144-150
7. Nishigaki, K., Husimi, Y., Masuda, M., Kaneko, K., and Tanaka, T. (1984) Strand dissociation and cooperative melting of double-stranded DNAs detected by denaturant gradient gel electrophoresis. *J. Biochem.* **95**, 627-635
8. Hutton, J.R. (1977) Renaturation kinetics and thermal stability of DNA in aqueous solutions of formamide and urea. *Nucleic Acids Res.* **4**, 3537-3555
9. Eli, P. and Husimi, Y. (1998) Imidazole improves specificity of PCR by suppressing the product-priming outgrowth. *Chem. Lett.* **1998**, 683-684
10. Gotoh, O. and Tagashira, Y. (1981) Stability of nearest-neighbor doublets in double-helical DNA determined by fitting calculated melting profiles to observed profiles. *Biopolymers* **20**, 1033-1042
11. Tachibana, H. and Wada, A. (1982) Ligand-induced melting reaction of specific-sequence DNA molecules. *Biopolymers* **21**, 1873-1885
12. Breslow, R. (1991) How do imidazole groups catalyze the cleavage of RNA in enzyme models and in enzymes? Evidence from "negative catalysis." *Acc. Chem. Res.* **24**, 317-324