Intramolecular DNA melting between stable helical segments: melting theory and metastable states

Ezra S. Abrams¹, Susan E. Murdaugh and Leonard S. Lerman*

Department of Biology, MIT, Cambridge, MA 02139, USA and ¹Pharmaceutical Peptides Inc, One Hampshire Street, Cambridge, MA 02139, USA

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ABSTRACT

Melting of DNA in a segment bounded at both ends by regions of greater stability during electrophoresis in denaturing gradient gels shows complex properties, not accommodated within standard melting theory. Compact bands of some DNA molecules become anomalously broadened at the retardation level in a denaturing gradient, or double bands may appear in a uniform denaturant concentration. These properties are associated only with molecules for which the distribution of stability calculated by the Poland–Fixman–Freire algorithms indicates that the region of lowest stability does not extend to an end of the molecule. Retention of helicity at the ends is shown by the difference in the effect of base substitution in the end domains and in the least stable domain. Both the appearance of double bands and band broadening can be explained by invoking a hypothetical metastable intermediate in melting, which is converted into the equilibrium melted form at a relatively slow rate, depending on both denaturant concentration and field strength. A kinetic model permits plausible rate constants to be inferred from the patterns. Despite the increased band width, sequence variants with base changes in the least stable domain result in readily detectable band shifts in the gradient.

INTRODUCTION

The stepwise melting of DNA molecules of length >100 bp in a gradually increasing denaturing environment is attributable to strong cooperativity and suppression of the unraveling of short interior loops or bubbles. The steps correspond to the concurrent melting within a narrow temperature interval of a large number of contiguous bases, called domains. Denaturing gradient gel electrophoresis (DGGE) is sufficiently sensitive to detect and measure the change in melting temperature of a domain resulting from substitution of a single base. It provides a general means for detection of gene mutations and polymorphisms.

Electrophoresis in a denaturing gradient differs from conventional gel electrophoresis in that the mobility of a fragment is not constant,

but rather drops abruptly as the fragment reaches a concentration of denaturant in the gradient that is characteristic for each fragment. We refer to the loss of mobility as retardation and to the denaturant concentration where it occurs as the retardation level. The close correlation between thermal stability calculated by Poland–Fixman–Freire algorithms and the observed retardation levels among many fragments permits us to identify retardation with partial melting, i.e. loss of helical structure in the least stable portion of the molecule (1). The drop in migration velocity on retardation produces a sharp band in the gel, often much sharper than the band formed after initial entry into the gel. Perturbations in melting temperature are recognized by a shift in the retardation level of a band in the gradient relative to that of a reference fragment. Band positions in the gradient and their displacement by substitution or mismatch are only weakly dependent on the duration of electrophoresis. The narrow band width contributes to the high resolving power of DGGE, permitting recognition of sequence effects as small as the addition of a single methyl group to either adenine or cytosine (2).

Although most unselected genomic and cloned DNA fragments reach a sharp focus at their characteristic retardation level, some molecules fail to focus, forming a broader band. These effects have been noticed in maize and *Drosophila*, as well as in human, genomic DNA. We have seen similar properties with some fragments of human genomic DNA. Broad banding may emerge when a short GC-dense sequence (termed a clamp) is added to one end of some fragments of human genomic DNA, although neither the original genomic fragment nor a similar construct with the same clamp sequence coupled at the opposite end exhibit broad banding.

The band distributions for three DNA fragments of identical sequence in the portion of the molecule with the lowest melting temperature but differing in flanking sequence are shown in Figure 1A, for several periods of running time. Lane 1 shows a typical, well-focused band; lanes 7 and 13 show easily recognized broad bands. The band widths in terms of the span of denaturant concentration at half the peak height are 0.2 and 1.1% respectively, an ~5-fold ratio. It will be useful to understand and to modify this property for efficient, exhaustive scrutiny of genomic sequences for the presence of variants or mutant sequence changes. To this end we have: (i) examined the theoretical stability maps and the base sequence of broad banding genomic fragments; (ii) made new constructs with predetermined stability patterns; (iii) explored

* To whom correspondence should be addressed

some variables controlling DGGE; (iv) developed a hypothetical mechanism that can account for the anomalous observations.

MATERIALS AND METHODS

Denaturing gradient electrophoresis was carried out as described previously (3,4). Linear denaturing gradient gels (8% acrylamide, 0.21% bisacrylamide, 1× TAE buffer) were run at 60°C unless otherwise noted. TAE contains 0.04 M Tris, 0.02 M sodium acetate, 1 mM disodium EDTA and is adjusted to pH 7.4. As previously, 100% denaturant is defined as 7 M urea and 40% (v/v) formamide (5). Gels (15 cm) were prepared with the Hoefer SE600 gel cassette, using 0.75 mm spacers; perpendicular gels were cast as described (3).

For gels in which the gradient is perpendicular to the direction of migration, restriction fragments are gel purified in low-melting agarose, since a band from the plasmid backbone in these digests would obscure the contour. Gel slices are mixed with loading buffer, melted at 60°C and applied directly as a continuous line across the top of the gel. After electrophoresis at 10 V/cm for 45 min at room temperature to bring the sample into the gel, the field is removed and gels are equilibrated at the desired final running temperature (usually 60°C) for 5–30 min. The voltage is then reapplied. The length of time allowed for equilibration does not affect the pattern. DNA was extracted from excised portions of these gels by electroelution.

Ethidium fluorescence patterns were either photographed or digitized directly with a CCD camera (Photometrics model 200, 1320 × 1320 pixel array), with subsequent image analysis on a UNIX workstation (Apollo Domain 10000) using programs written in this laboratory. In some cases photographic negatives were digitized with an Eikonix model 2000 photodiode array camera, with subsequent image analysis.

Plasmids designed to carry end- and middle-melting sequences were constructed using the GC clamp vectors pCH6, pCH14 and pCH1 (3,6); these plasmids have GC clamps of 30, 60 and 150 bp respectively in the *HincII* site of pTZ19U (7). Three plasmids (pMM4, pMM5 and pMM9) with anonymous *HindIII*–*PstI* fragments were isolated from germline human DNA cloned into *HindIII*/PstI-cut pCH6 or pCH14. The *HindIII*–*EcoRI* fragments from these plasmids contain the clamp sequence adjacent to the human *HindIII*–*PstI* fragment. Mutations were introduced into the *HindIII*–*EcoRI* fragment of pMM4 either by oligonucleotide-directed mutagenesis (pMM4m3 or pMM4m4) or by mutagenic PCR (pMM4m1 or pMM4m2); specificity of mutagenesis was checked by resequencing of appropriate regions. Plasmid p408 has a 408 bp *PstI* fragment from the human EPO gene in the *PstI* site of pCH14, resulting in a 60 bp clamp adjacent to the promoter proximal end of the EPO fragment; in p408-2 the EPO fragment is inserted in the *PstI* site of pCH1, resulting in a 150 bp GC clamp at the 5'-end of the fragment. Plasmid p408-3 has the EPO *PstI* fragment with 150 bp GC clamps at both ends. Plasmids pCH6G1 and pCH14G1 (6) have a 272 bp *HaeIII* fragment spanning the first intron of the human globin gene cloned between the *PstI* and *HindIII* sites of pCH6 and pCH14. Plasmid pCH14Gs1 has a 340 bp *Sau96I* fragment spanning the cap site of the human β -globin gene cloned into pCH14 (6).

The distribution of thermal stability along each molecule was computed using our program MELT (8), which is based largely on the algorithm of Fixman and Freire as a computationally practical

Figure 1. Bands formed by end- and middle-melters in parallel DGGE shown as a function of running time. (A) Restriction fragments consisting of the 408 bp *PstI* fragment from the human EPO gene, with a clamp on the 3'-end, end-melting (lanes 1–6), a clamp on the 5'-end, middle-melting (lanes 7–12) or a clamp on both ends, middle-melting (lanes 13–18) were applied to a 40–60% parallel gradient run at 10 V/cm, 60°C. Samples were loaded at intervals to provide the desired duration of exposure to the field: lanes 6, 12 and 18 had 3 h running time; the others, from right to left in each group, were given successive 1 h increments up to 8 h in lanes 1, 7 and 13. The band at the top of the gel is the plasmid backbone. (B) Meltmaps for the restriction fragments used in (A).

Figure 2. Melting maps for three anonymous restriction fragments of human genomic DNA with added GC clamps that form broad bands in parallel gradients. (A) The *EcoRI*–*HindIII* fragment from pMM4. Sites of substitutions after *in vitro* modification making pMM4m1 (bp 171, GTA→GGA), pMM4m3 (bp 223 CTT→CCT) and pMM4m4 (bp 245, GTT→GGT) are indicated. The fragment from pMM4m2 differs from pMM4 by four base substitutions in the downstream half of the low-melting domain (bp 90, CTT→CCT; bp95, CAT→CTT; bp144, TTT→TCT; bp164, AAG→AGG). Its melting map differs in only that domain, as indicated by the dashed line. (B) The *EcoRI*–*HindIII* fragment (solid line) and the *PstI*–*HindIII* fragment (dashed line) of pMM9. (C) The *EcoRI*–*HindIII* fragment of pMM5.

approximation of Poland's sequence-specific, two-state theory of the helix–random chain transition (9,10).

RESULTS

Association of calculated melting properties with broad bands

Fragments of ~300 bp that are retarded as broad bands after addition of a GC clamp were found in a library of human genomic DNA. The base sequence was determined for three of these fragments (melting maps are shown in Fig. 2A–C). Melting maps for three different naturally occurring molecules that focus to sharp bands, arbitrarily selected from a large collection from diverse sources, are shown in Figure 3A–C.

The distinguishing feature between poorly focusing and sharply focusing molecules is that in the former the domain responsible for retardation in the gel lies in the interior of the fragment, not extending to an end. As these molecules migrate into the denaturing gradient, sufficient melting to substantially reduce the migration velocity would be expected to begin somewhere within the sequence, rather than at one end. Conversely, when the domain with the lowest calculated T_m is at an end (Fig. 3A–C) the fragment forms a sharp band, as noted in previous studies (5). The presence of a short sequence of low thermal stability at the end of a molecule

Figure 3. Melting maps of human DNA fragments forming sharp bands, arbitrarily selected from a large supply. (A) The *EcoRI*–*HindIII* fragment from pCH14G1. (B) The *PstI* fragment from p408. (C) The *PstI*–*HindIII* fragment from pCH14Gs1.

(appreciably fewer than 75 bp, the length giving $1/e$ velocity reduction) has no effect on either type of molecule, other than a small lowering of the initial velocity.

The association of broad banding with middle-melting was tested by assembling constructs from component sequences of predictable stability. A 408 bp *PstI* fragment from the EPO gene was cloned into different plasmids such that restriction fragments consisting of the *PstI* fragment with a GC clamp at one or other or both ends could be obtained. The melting maps shown in Figure 1B correspond to these constructs. Their gradient properties are shown in Figure 1A. Molecules carrying a GC clamp at the 5'-end (lanes 7–12) or at both ends (lanes 13–18) give broad bands, while the fragment with a clamp only at the 3'-end gives a narrow band (lanes 1–6). Numerous other molecules have shown the same properties.

Retardation and band formation

The kinetics of transport in the gradient and the anomalous properties of central-melting molecules are displayed in sequential examination of bands as they migrate. Four sets of band profiles in Figure 4 show progress through a gel for both an end-melting molecule (upper pair) and a middle-melting molecule (lower pair) carrying the same least stable domain migrating at two different field strengths (left pair, 10 V/cm; right pair, 1.3 V/cm). Melting maps, which show the equilibrium temperature at which each base pair in the sequence is equally distributed between the helical and melted states, are shown in Figure 5. At high field strength the band positions were followed at hourly intervals after application of the field. At low field strength the same molecules were followed at 6 h intervals. A number of significant features can be discerned:

(i) The incremental advance of each band through uniform time intervals declines as the band moves through the gradient.

(ii) The band profile of the end-melter remains sharp at both values of field strength. Some sharpening can be seen at low field strength. This effect would also have been evident at high field strength if the initial band had been broader.

Figure 4. Retardation and band profiles where a low-melting domain is bounded on only one or both sides by higher melting domains, at two field strengths. Meltmaps for these fragments are shown in Figure 5. The *PstI*-*KpnI* fragment, end-melter (top pair) and the *HindIII*-*KpnI* fragment, middle-melter (bottom pair) from pALS2 were electrophoresed on 25–65% parallel gradients for 5 h at 10 V/cm (left, top and bottom) or 36 h at 1.3 V/cm (right, top and bottom). Fluorescence after ethidium staining was recorded digitally with a CCD camera. A single scan line for each lane is shown.

(iii) The band of the middle-melter in the stronger field (lower left) spreads as it is retarded to form a broad band.

(iv) Spreading of the middle-melter on retardation is also seen at low field (lower right), but is less pronounced.

(v) The middle-melter proceeds a little further into the gradient than the same low-melting domain as an end-melter before it is fully retarded.

What bounding increment in stability is needed to define a middle-melting domain?

For molecules with interior lowest-melting domains, we ask whether a bounding domain calculated to have only a few degrees greater stability remains helical when the interior domain melts. We have shown that the boundaries of intramolecular melting can be precisely localized by determining whether a base substitution (or mismatch) shifts the retardation level in a denaturing gradient (11,12). The retardation level is unaffected by substitutions in a region that remains helical, even though that region melts at only a slightly higher temperature. Substitutions were generated in a broad banding molecule, the *EcoRI*-*HindIII* fragment of pMM4, indicated in Figure 2A. The low-melting tail at bp 380–430 is too short to effect retardation (1). Retardation levels in the gradient are shown in Figure 6, where it can be seen that displacements are easily discernable, despite the breadth of the bands. The data in Figure 6 show that substitutions in the middle-melting domain of pMM4 (T_m 67.5°C) give a displacement, while no displacements result from either of the two substitutions in the 70.2°C bounding domain. The site of the substitution (at bp 171) furthest toward the 3'-end of the 67.5°C domain lies only 9 bp from the domain boundary. Since no band shifts are seen from substitutions in the bounding domain at 180–310 bp, we infer that the 70.2°C region remains helical when melting of the 67.5°C domain effects

Figure 5. Meltmaps of the fragments described in Figure 4.

retardation and that the theoretical maps offer a reliable description of the distribution of thermal stability.

Voltage-sensitive patterns seen with perpendicular DGGE

The relations between denaturant concentration and electrophoretic mobility are analyzed both more simply and in greater detail with data taken from a perpendicular gradient gel, where each molecule travels at constant velocity along a path of uniform denaturant concentration. The gel, which carries a broad span of denaturant

Figure 6. Correspondence between band shift and map position of a mismatch site in middle-melting molecules. The *EcoRI*–*HindIII* fragment from pMM4 or pMM4 mutants were analyzed on a 27–54% parallel gradient run at 6.7 V/cm, 60°C for 10 h. Lanes 1–5: heteroduplexes were formed between pMM4 and mutant fragments by heating and slow cooling; lane 1, pMM4 by itself; lane 2, pMM4 + pMM4m1 (mismatch at bp 171); lane 3, pMM4 + pMM4m2 (four mismatches, bp 90, 95, 144 and 164); lane 4, pMM4 + pMM4m3 (mismatch at bp 223); lane 5, pMM4 + pMM4m4 (mismatch at bp 245). Lanes 6–9: PCR fragments after digestion with *HindIII*/*EcoRI* (no heating); lane 6, pMM4m1; lane 7, pMM4m2; lane 8, pMM4m3; lane 9, pMM4m4. Lane 10: control end-melter digest of pRAS3-24. The retardation levels of heteroduplex restriction fragments (HETS) and homoduplex restriction fragments (HRF) are indicated.

concentration orthogonal to the direction of migration, provides a clear representation of velocity as a function of denaturant concentration. Figures 7 and 8 show the perpendicular gradient patterns from end- and middle-melting molecules (meltmaps shown in Figs 3A and 2B) determined by migration at 10 (high field) or 1.3 V/cm (low field). The starting reference level is a little below the top of the gel (see Materials and Methods). For the end-melter at 10 V/cm (Fig. 7A) high migration velocity at low denaturant concentration is seen at the left. There is an abrupt transition to extremely low velocity at a critical gradient level near the center of the gel and a second transition to the greater mobility and smearing characteristic of fully separated single strands at higher denaturant.

While there are minor differences between the end- and middle-melters for perpendicular gels run at 10 V/cm, (Figs 7A and 8A), the patterns show the same sharp mobility transition and we infer that retardation by middle-melting does not differ greatly from that of end-melting at this field strength.

In a weaker field (1.3 V/cm) conspicuous differences appear, as shown in Figure 7B for the end-melter and Figure 8B for the middle-melter. Figure 8B shows that the middle-melter gives two distinct bands over the gradient interval between the beginning of the transition and full retardation. The upper band appears to be fully retarded over the interval, while the velocity of the rapidly migrating band decreases gradually with increasing denaturant concentration. As seen in Figure 8B, the faster contour shows that an ~8% increment in denaturant concentration, equivalent to an ~4°C temperature increment, is required for the mobility to decline to 20% of its value before melting and an additional 10% increment in denaturant, or 5°C in equivalent temperature, is required for full melting. The pattern in Figure 8B, where formation of two distinct bands in a perpendicular gel run at 1.3 V/cm is associated with calculated middle-melting, is typical of results obtained with many different fragments.

Figure 7. Electrophoretic velocity versus denaturant concentration in a perpendicular gradient for an end-melting fragment at high field strength and at low field strength. The *EcoRI*–*HindIII* fragment from pCH6G1 was applied as a continuous line across the top of a 30–90% perpendicular gradient and driven into the gel at room temperature for 30 min before exposure to the 60°C bath. An electric field was then applied at (A) 10 V/cm for 3.75 h or (B) 1.3 V/cm for 22 h. This fragment has a 30 bp GC clamp and the meltmap is nearly identical to the fragment with a 60 bp GC clamp (Fig. 3A).

We have excluded the possibility that two bands are formed because the starting material contains two or more distinct species or that irreversible modification occurs during electrophoresis. The *XbaI* fragment of p408-2 (this fragment is similar in gradient properties to the *KpnI*–*HindIII* fragment of p408, Fig. 1B) was end-labeled with ^{32}P . The ^{32}P -labeled fragment gave a two-band pattern in the perpendicular gradient. DNA was extracted from two gel sections excised at the positions of the upper and lower bands and applied to similar gradients. Both excised samples reproduced the same double contour as the original. Therefore, the double band is attributable to a single species and there is no irreversible change in the molecules. This also suggests that the phenomenon is entirely intramolecular and independent of DNA concentration, in that the amount of DNA in the secondary gel is much lower than that of the original, but duplicates the same pattern.

Since the partly melted molecules contain a fully closed, single-strand loop, it might be supposed that the fully retarded, upper

band represents some fraction of partially melted molecules that have been trapped by the gel structure, analogous to the impaling of double-strand circles on agarose spurs proposed by Levene and Zinn (13). However, end-melting molecules are just as highly retarded without loops. The hypothesis offers no means of explaining why those molecules that escape trapping at the start of the low field run continue to escape trapping as they proceed further through the gel. We have failed to find sharpening of broad bands at 1.3 V/cm using a reversing field, which has been shown to liberate impaled molecules, using periodicities down to 3 s forward, 1 s back (13).

Effect of adding a GC clamp to both ends of a fragment

Adding a GC clamp to the unclamped end of the middle-melter fragment from p408-2 (gel, Fig. 1A, center; meltmap, Fig. 1B, center), to give a fragment with 150 bp of GC-dense sequence at both ends (gel, Fig. 1A, right; meltmap, Fig. 1B, bottom), does not alter the calculated melting properties of the lowest-melting middle domain and the two fragments show similar retardation levels in a parallel gel run at 10 V/cm (Fig. 1A, lanes 7 and 13). However, in a perpendicular gel run at 1.3 V/cm (Fig. 9), the fragment with one GC clamp shows the two-band pattern (Fig. 9A), while only a single band is seen within the same span of gradient for the fragment with two GC clamps (Fig. 9B). Similar results were obtained with a set of middle-melting fragments with one or two GC clamps, where the low-melting domain is derived from a *HinfI* fragment from the human β -globin gene.

DISCUSSION

What molecular features pertain to broad banding?

Among unselected DNA molecules of 150–600 bp we find experimentally that there are two nearly discrete classes with respect to the breadth of retarded bands; most retard as sharply focussed bands, while some retard as broad bands. When sequenced we find that all representatives of these classes are also distinguished by their theoretical patterns of thermal stability, the meltmap, according to the Poland–Fixman–Freire algorithms. In molecules that form broad bands the part of the sequence with the lowest thermal stability is both preceded and followed by regions of higher thermal stability. In molecules that come to a sharp focus the region of lowest thermal stability extends to an end of the sequence. This correspondence between band width and the position of the calculated lowest-melting part of the molecule is maintained over a large set of different, unrelated sequences, in both naturally occurring molecules and appropriately designed constructs. These appear to have no detailed features in common except the calculated stability pattern.

Since genomic and cloned or amplified fragments behave similarly, the band width cannot be attributed to base modifications. A- and T-dense tracts are found in both end- and middle-melters; only some of the broad banding fragments carry significantly long runs of A or T. Details of the nature of the more stable ends are unimportant; the high GC density of clamp sequences is not necessary. The single relevant feature appears to be the pattern of thermal stability inferred from sequence by means of the theory of the helix–random chain equilibrium.

Among a small sample of fragments the extent of band broadening correlates with the difference between the T_m of the lowest-melting domain and that of the nearest bounding domain. For the three fragments shown in lane 3 of Figure 6 the breadth of

Figure 8. Velocity versus denaturant concentration in a perpendicular gradient for a middle-melting fragment, the *EcoRI*–*HindIII* fragment of pMM9. Same procedure as in Figure 7. (A) 10 V/cm, (B) 1.3 V/cm. The meltmap appears in Figure 2B.

the band increases in the order pMM4m2 homoduplex < pMM4 homoduplex < heteroduplex. For pMM4 the difference in T_m between the lowest-melting domain and the bounding domain is only 2°C (Fig. 2A); for pALS2 (Fig. 5B) the difference is ~13°C. pMM4 forms bands less conspicuously broad than pALS2, which forms very broad bands.

Do the theoretical domain boundaries reflect real discontinuities in thermal stability?

These results bear on the localization of melting as predicted by theory in a way that has had little prior challenge. The only high resolution tests of melting location have depended on the perturbation of melting by base substitutions or mismatches at a series of closely spaced positions defined by base sequencing. Fisher and Lerman showed that a series of base substitutions within the region of a fragment of λ DNA shown by theoretical calculation to have the lowest thermal stability altered the melting properties of that region, while nearby substitutions in a region calculated to have a higher stability did not alter the melting of the least stable region (11). The calculated boundary between the domains of lower and higher thermal stability in the fragment

Figure 9. Velocity versus denaturant concentration in a perpendicular gradient: effect of an additional, high-melting domain on a middle-melter. (A) The *KpnI*-*HindIII* fragment of p408-2, mapped in Figure 1B; (B) the *KpnI*-*HindIII* fragment of p408-3, also mapped in Figure 1B, was constructed by the 3' addition of a 150 bp clamp to the fragment of p408-2. Gel conditions: 30–90% gradient, 60°C, 1.3 V/cm, 22 h.

matched the interval between perturbing and non-perturbing substitutions within ± 1 bp.

In the present study the absence of band displacement accompanying a base substitution within the sequence of a bounding domain with a predicted stability only 2°C higher than that of the predicted least stable, central domain supports the calculated meltmap as a description of local sequence stability where melting generates a bounded loop.

Broad bands and melting theory

Melting theory provides no clue as to the origin of anomalous band widths. Aside from the map contour, features of the helix-melt equilibrium given by the theory are not significantly different between molecules in which the lowest-melting domain extends to an end and molecules in which the lowest-melting domain is bounded by domains lying at higher temperatures. As seen in Figures 2 and 3, the predicted middle-melting domains are as fully flat and sharply bounded as typical end-melting domains. The calculated breadth of the temperature interval required for the transition is comparably narrow for both.

To examine the relation between melting and mobility more closely, we have used the perpendicular gradient system, in which each molecule migrates along a path of uniform denaturant concentration and the effect of a broad range of denaturant concentration is displayed. The contours across the gradient represent the mobility versus temperature function (uncorrected for the changing viscosity of the medium), which is determined by the calculated total number of bases melted as a function of temperature. For end-melting molecules the calculated transition of a domain from 80 to 20% helicity typically spans a small temperature interval, $\sim 1^\circ\text{C}$. That steep transition corresponds to the abrupt decline in mobility seen at high field strength, where the increment in denaturant concentration is too small to infer from the gel pattern. Similar small calculated and experimental intervals for the transition are found with the middle-melting fragment shown in Figure 8A at 10 V/cm. The sharp transition is essentially the same for middle- and end-melters, even though retardation depends on a looped out segment, rather than on unraveled free ends.

Non-equilibrium effects

Comparison of calculated melting properties and experimental contours in perpendicular gradients is more complex at low field strength. Rather than a single curve in the gel, middle-melters usually form two contours, one strongly retarded after a sharp transition at a critical denaturant concentration, as expected by calculation, together with a more rapidly migrating, curved band (Fig. 8B). The contour with a mobility that declines gradually with increasing denaturant concentration is also seen with some end-melting molecules at very low field strength, but the double contour has not been observed.

From the double contour we infer that the initial sample rapidly divides into two forms of different structure. Since the same pair of bands formed again from the DNA samples recovered separately from the rapid and retarded forms in a previous gel showing the double contour, it cannot be supposed that there are two distinct species differing in sequence or bond continuity. Band splitting can be attributable to isomerization, an interchange between multiple forms of the same molecule. However, the pattern given by isomerization is critically sensitive to forward and backward rates. Only if the rates are exceedingly slow will two distinct bands be expected. If the rates are very fast relative to migration velocity, a single, well-defined band of intermediate properties is expected and under some conditions all three bands appear. Variation of the rate of heating and the initial voltage are without effect. Since two forms persist through prolonged electrophoresis but return to the same initial state on cooling, equilibrium isomerization is excluded and we infer that one of these structures is metastable under the conditions of the experiment.

The wide, continuous variation of migration velocity of the rapid band at low field strength spreads over a broad span of denaturant concentration. The drop from 80 to 20% of the initial velocity requires an increment in denaturant equivalent to a temperature interval of 7°C or more. This gradual change lacks any counterpart in two-state melting theory, which is indifferent to field strength and predicts a drop from 80 to 20% helicity over $\sim 1.0^\circ\text{C}$. The gradual change in mobility suggests the participation of a set of intermediates with structures that adjust to the denaturing environment, rather than a single, sharply defined structure. However, some of the adjustable structures that are easily imagined, for example cruciform, H-forms

or loops of varying stem length, have special sequence requirements not seen in our molecules.

The fully retarded branch of the contour was not seen in the perpendicular gradient pattern for a molecule carrying 150 bp GC-dense sequences at both ends (Fig. 9B). However, the denaturant level for the onset of that contour is sequence-dependent and it is likely that the appearance of the slow band requires a higher level of denaturant.

Kinetics and stability patterns

It has been recognized within two-state melting theory that there can be large differences in the rates of helix formation within a partly melted molecule not too far from equilibrium, depending on the state of the contiguous sequence. (i) A melted, low stability domain extending from the helical end is expected to helicize rapidly, since the existing helix serves as a structural nucleus. (ii) Very slow helicization is expected for a molecule with a middle-melting stability pattern if an end should be melted as well as the middle domain. As the temperature falls, helix must form first at the end domain, but it is separated from the existing helix at the other end by the melted middle domain. The helicization rate of the end domain will be limited by the probability of a productive encounter between the melted ends. The $t_{1/2}$ of such nucleation events may be comparable to the time of electrophoresis ($t_{1/2}$ of the order of 100–1000 s) (14,15). The failure of substitutions in the end domain to effect band displacements (Fig. 6) shows that the end domains are not melted and that this mechanism does not apply to the present system. (iii) Where a melted domain is bounded at both ends by helix, as in a middle-melter, the expected rate for helicization of the unconstrained molecule is the most rapid of all. Thus we are unable to account for slow equilibration by known structures or mechanisms.

The origin of band broadening

We propose a mechanism for band broadening in parallel gradients that also accounts for the double bands at low field strength in constant denaturant, based on the mobility of the metastable intermediates and their rate of conversion to the equilibrium form. It is necessary to assume that the rate of conversion depends on field strength and denaturant concentration. In parallel gradients those molecules that escape immediate full retardation at the normal level adopt the metastable structure and continue to advance beyond that level, although at a reduced rate. While they advance they undergo some slow conversion to the fully retarded, equilibrium form and the residual band of the more mobile metastable form continues on until it is depleted by the conversion. The products of conversion are fully retarded, but strung out over the interval the intermediate moved from the start of conversion until its exhaustion; their continued, slow migration preserves the relative depths at which conversion took place. The rate of conversion can be expected to rise sharply as a function of denaturant concentration.

As a unimolecular transformation, the fractional rate of loss by conversion from the metastable band can be specified by an assumed relation of the rate to gradient level. Where x is the amount of DNA at each point in the band and y is the increment in denaturant concentration above the calculated retardation level, the fractional rate is $(dx/dt)/x$. If the fractional rate of loss per unit of travel is taken to increase as the first or second power of y

Figure 10. Simulation of broad bands. The broad bands were calculated from equations 3 and 4. A single parameter, the ratio of the rate constant to the velocity of the hypothetical intermediate, kE/v , was selected to provide a contour approximately matching Figure 4, lower left. The conversion rate increases from a negligible value at the nominal (or end-melting) retardation level, 0.0 in the plot, according to the first (solid line) or second power (dashed line) of the gradient increment. The broader, right-hand curves were calculated for the value of kE/v corresponding to Figure 4; the narrower, left-hand curves are based on a 5-fold larger value of kE/v . All profiles are adjusted to unit peak height, rather than constant DNA amount. The sharp profile (dotted) centered at 0 shows the approximate band width of typical end-melting molecules.

$$(dx/dy)/x = -kE/v y \quad 1$$

or

$$(dx/dy)/x = -kE/v y^2 \quad 2$$

$X(y)$, the distribution of DNA in the final, broad band is the amount lost from the intermediate band; thus

$$X(y) = -(kE/v)y e^{-(kE/2v)y^2} \quad 3$$

or

$$X(y) = -(kE/v)y^2 e^{-(kE/3v)y^3} \quad 4$$

where the variables kE (/h) and v (cm/h) denote values for the rate constant, assumed to be a function of the field strength, and the migration velocity. The increase in the rate constant with increasing field strength will account for the observation of double bands only at low field strengths.

The hypothesis permits comparison between experiment and calculation with respect to both the breadth of bands and the shift in level of full retardation relative to end-melting molecules by evaluation of a single parameter, kE/v . The shifts, y_1 and y_2 , of the maxima of broad bands given by equations 3 and 4 are

$$y_1(dx_{\max}) = (v/kE)^{1/2} \text{ cm} \quad 5$$

and

$$y_2(dx_{\max}) = (2v/kE)^{1/3} \text{ cm} \quad 6$$

respectively. The shift in Figure 4 is 3.2% denaturant, ~1.2 cm into the gel. Taking an average, uniform velocity, v , for the metastable form as ~1/2 of the helical form, 1.0 cm/h, the rate constant for conversion, kE , is ~0.70/h under the first power model, which is fast enough to interfere with observation of the intermediate in perpendicular gels at high field strength. The second power model gives 1.16/h. Assuming that the rate is proportional to the field strength, the rate at 1.3 V/cm in the first power model is only 0.091/h, implying a lifetime of 11 h at low field strength, long enough to generate the double bands seen in Figure 8B. The value

for the second power is 0.151/h. The corresponding calculation for the band shift of fragment p408-2 shown in Figure 1 gives rate constants for conversion of 1/4 or 1/8 of the preceeding values.

A plot showing the shift and spread of a fully retarded band is shown in Figure 10 for both the first and second power models (assuming negligible width before retardation) for two values of kE/v , together with a profile of a normal band (dotted), retarded at the level of sharp melting. The right-hand, broader curves in Figure 10 were calculated for a value of kE/v giving a band shift of 3.2% denaturant, corresponding to the 10 V/cm profile in Figure 4, solid for equation 3, dashed for equation 4. The left-hand, narrower curves were calculated with a 5-fold larger value of kE/v . In agreement with the result in Figure 4, lower right, the calculation predicts less broadening at lower field strength (a larger value of kE/v).

The band width calculated from the shift and the inferred rate constant remains consistent with the data in Figure 4. The width of the fully retarded 10 V/cm band at half the peak height in Figure 4 is ~4% denaturant, just between the calculated widths at half maximum for the two models, 3 and 5%.

Specific proposals on the nature of the hypothetical intermediate structure and an explanation of its low rate of equilibration await further study. We have not been able to discern sequence regularities, such as palindromes or direct repeats, that might suggest recognizable structures. There are polypyrimidine tracts, but these are short. Furthermore, the intermediate cannot represent a unique structure; its gradual decline in mobility across the gradient implies a nearly continuous variation in form or a continuous shift in rapid equilibrium between two different anomalous forms. It seems that the intermediates inferred here are not the same as the metastable intermediates proposed by Suyama and Wada to account for multiphasic kinetics observed in temperature jump studies on many-fold longer molecules of *ColE1* DNA (15). Similarly, the relatively slow processes reported for T2 and T7 DNA were seen in long molecules (16). We cannot exclude the participation of corresponding intermediates in end-melting molecules, providing that they exhibit a much shorter lifetime.

The similarity between the gradual contours of the rapidly migrating component of the middle-melters and the contours of some end-melters at very low field strength suggests that structures similar to the hypothetical metastable types of middle-melters are formed, but with a shorter lifetime.

The role of field strength

The field strength effect suggests that there is a direct interaction of the field with melting, destabilizing intermediate forms, perhaps based on the greater net charge of separated strands. If so, the mechanism of the field effect is subtle, since there is no distinct perturbation in the denaturant level for equilibrium melting of the first domain.

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