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SEPARATION OF LARGE DNA MOLECULES BY PULSED-FIELD GEL ELECTROPHORESIS

A REVIEW OF THE BASIC PHENOMENOLOGY

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SUMMARY

Pulsed-field gel electrophoresis is a method of separating large DNA molecules. The distinctive feature of this method is that the direction of the electric field is changed periodically. During the five years since Schwartz and Cantor introduced this technique, there has been dramatic progress in pulsed-field instrumentation and in associated electrophoretic methods. Progress has been driven by practical experience with little guidance from theory. In this review, the basic phenomenology of pulsed-field gel electrophoresis is summarized and some speculations are advanced about possible molecular mechanisms.

INTRODUCTION

Pulsed-field gel electrophoresis (PFGE) is a method of separating large DNA molecules that was introduced by Schwartz and Cantor in 1983[1]. In its usual forms, it involves alternately applying two electric fields that differ in direction. Electrophoretic conditions are otherwise similar to those normally used to separate double-stranded DNA molecules ranging in size up to tens of thousands of base-pairs. By the simple device of periodically alternating the electric field direction, the size range for separations can be extended to several millions of base-pairs. The time scale of the alternations is typically in the range of seconds to minutes, increasing with the sizes of the molecules that are to be separated.

Under typical PFGE conditions, the mobilities of small DNA molecules (*i.e.* those ranging in size up to 10 000–15 000 base-pairs) are not affected by the switching events. These molecules simply migrate in accordance with the time-averaged electric field. However, larger DNA molecules behave quite differently. During continuous electrophoresis, they adopt an elongated conformation and their long axes become aligned with the electric field. The motion of such molecules through the gel has been referred to as "reptation" since it resembles the forward motion of a snake. In continuous electric fields, the mobilities of reptating molecules larger than a threshold size (typically, 20 000–30 000 base-pairs) are independent of size. In PFGE, however, the mobilities of these molecules are diminished compared to the continuous-field case.

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Because the magnitude of this effect is a sensitive function both of the size of the molecules and the frequency of the switching events, molecules that are altogether inseparable by continuous electrophoresis can have dramatically different mobilities in PFGE.

During its short history, PFGE has been carried out on a succession of apparatuses that differ primarily in the shapes and relative orientations of the two alternately applied electric fields. The first successful separations employed an apparatus in which at least one of the alternately applied electric fields was spatially non-uniform. Because of the field inhomogeneity and the orientation of the wells relative to the applied fields the molecules migrated along curved trajectories[1,2]. In order to emphasize the presence of large gradients in the applied fields, the method was referred to as pulsed-field-gradient (PFG) electrophoresis. Carle and Olson[3] introduced an apparatus in which —at least in the central lanes— the molecules migrated in a straight line. However, this line was defined by the time-averaged direction of two symmetrically applied, inhomogeneous fields; consequently, trajectories were still sharply curved towards the edges of the gel, albeit in a symmetric pattern. The name orthogonal-field-alternation gel electrophoresis (OFAGE) was suggested to accentuate the spatial relationship between the alternately applied fields.

A number of perplexing features of the phenomenology were described in these early papers, particularly the apparent need for inhomogeneous electric fields. Nonetheless, the observations were basically consistent with a "corner-turning" model. In this model, the dramatic size-dependence of the mobilities of large DNA molecules on pulsed-field gels was thought to occur because large molecules take longer to turn corners in the gel than do small molecules.

The first indication that neither inhomogeneous electric fields nor corner-turning were central to the pulsed-field effect came with the discovery that periodic inversion of a uniform electric field also gives rise to strongly size-dependent mobilities[4]. In field-inversion gel electrophoresis (FIGE), net forward migration is achieved either by employing longer switching intervals or higher field strengths in the forward than in the reverse direction. The optimum switching intervals for separating molecules in a particular size range are strikingly similar to those observed in the earlier experiments with transverse alternating fields, an observation that suggests that the molecules are undergoing similar types of rearrangement in the two cases.

The most recent phase of instrumental development involves a field geometry similar to OFAGE, but featuring uniform fields. The key to success in achieving OFAGE-style separations with uniform fields —and, thus, straight-line migration all the way across the gel— was the recognition of a requirement that the fields intersect at a markedly obtuse angle[4–6]. Earlier unsuccessful experiments with transverse homogeneous fields had all evidently been carried out at 90°, which any simple corner-turning model would predict to provide the greatest effect. In actuality, there is a strong requirement for an obtuse angle. Several types of instrumentation, such as the contour-clamped homogeneous electric field (CHEF) system[6], which is based on a closed hexagonal array of electrodes, and table-turning apparatuses[7,8], in which the gel rotates in the presence of a stationary electric field, can provide uniform alternating electric fields that intersect at an obtuse angle. The angle does not appear to be particularly critical, as long as it is significantly greater than 90°; a value of approximately 120°, as in the CHEF system, is highly effective.

In summary, two basic electrophoretic geometries arose during the early years of PFGE, and they continue to dominate current practice. In one of these geometries (FIGE), the alternating electric fields and the net movement of the molecules are all colinear. The other geometry involves two alternating fields that are transverse to one another and also to the direction of net migration. Systems of the latter type will be referred to here by the generic acronym TFAGE for transverse-field-alternation gel electrophoresis. As this acronym suggests, the basic geometry of TFAGE systems is similar to that of OFAGE. However, OFAGE was a misnomer, since it is known that an important feature of the OFAGE geometry was that the fields were not orthogonal: as in all successful TFAGE systems, they intersected at an angle greater than 90°.

DNA molecules behave quite similarly in all TFAGE systems. Although instrumental advances have been of great practical importance, particularly in providing straight lanes, they have not revealed any basically new electrophoretic effects. FIGE, on the other hand, does involve electrophoretic behavior that is qualitatively different from that observed with TFAGE. The most notable FIGE-specific effect is the presence of a sharply double-valued relationship between size and mobility[4]. Molecules of some intermediate size, which can be selected by tuning the frequency of the field inversions, typically have zero mobility. The mobilities of smaller molecules are inversely related to their sizes (as on a conventional gel), while the mobilities of larger molecules actually increase with increasing size. The latter phenomenon is weakly displayed on TFAGE[9] —and has been observed even on ordinary agarose gels under extreme conditions[10]— but it is a dominant feature of FIGE separations over a broad range of sizes, field strengths, and switching conditions[4,11].

Several efforts have been made to analyze PFGE from a theoretical perspective. These efforts span a wide range of formalisms, ranging from extensively developed theories based on the statistical mechanics of macromolecules[11] to entirely heuristic models[7]. Although these treatments offer a variety of insights into molecular processes that may be important in PFGE, they all have severe limitations. For example, the adaption of the "biased reptation" theory to FIGE by Lalande *et al.*[11] fails to explain the sharp minimum in FIGE mobility as a function of DNA size and also incorrectly predicts that the FIGE effect will disappear when the forward and reverse field strengths are equal. The heuristic model of Southern *et al.*[7] provides a possible explanation of the need for obtuse angles in TFAGE, but does so in a way that offers no insight at all into FIGE.

UNIFIED VIEW OF PFGE PHENOMENOLOGY

Given the complexity of the problem, progress towards a powerful, unified theory of PFGE is likely to be slow. In the absence of such a theory, it would at least be useful to have a systematic way of thinking about pulsed-field phenomenology, particularly if all the prominent features of both TFAGE and FIGE could be accomodated. Starting with ideas that were briefly developed in the original description of FIGE by Carle *et al.*[4], an effort will be made here to develop a highly simplified, but unitary, view of TFAGE and FIGE phenomenology.

The starting point is the concept that DNA adopts a directional conformation during ordinary steady-state electrophoresis. That is, an electrophoresing DNA molecule that is much larger than the size of the pores in the gel should not be regarded as

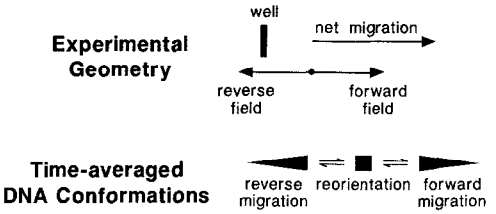

Fig. 1. Field-inversion gel electrophoresis (FIGE). The top of the diagram indicates the simple electrophoretic geometry. Note that the forward and reverse fields can be of equal strength with longer forward than reverse switching intervals, the switching intervals can be equal with a higher forward than reverse field, or some combination of inequalities in the field strengths and the switching intervals can be used to achieve net forward migration. The bottom of the diagram presents a simple three-state model for the conformational changes that accompany the field-inversion events.

a symmetric rod. A closer approximation would be a cone, represented in two dimensions as a wedge. While a rod would be expected to slide easily back and forth in an inverting field —responding only to the net forward impulse— the wedge is unable to change directions without undergoing a conformational change, which will be referred to as wedge inversion. The rate of wedge inversion is hypothesized to be slow and strongly size-dependent, the rate decreasing monotonically as the size of the molecules increases. Consequently, the simplest possible model for FIGE would involve three states, two of which are physically identical but oppositely oriented wedges (Fig. 1). Under particular electrophoretic conditions, the interconversions between the three states are assumed to be first-order kinetic processes. Furthermore, the mobility of the intermediate conformation, represented as a square, is assumed to be zero in either direction, while the mobility of an inappropriately oriented wedge is also assumed to be zero.

This simple formalism is adequate to explain basic FIGE phenomenology, particularly the dramatic double-valuedness of the size–mobility curve. When the time scale of the switching is closely matched to the rate of conformational interconversion, the molecules spend most of their time in the intermediate, zero-mobility conformation. We have previously described this phenomenon as "resonance" since it is a singularity that arises when the frequency of a driving force (the electric field) is tuned to a basic molecular parameter (the rate of conformational interconversion)[4]. Molecules much smaller than those at resonance have high mobility, since they can respond instantaneously to the field inversions: the ratio of time spent in the forward conformation to that spent in the reverse conformation is simply the ratio of the forward to the reverse switching intervals. On the other hand, molecules much larger than those at resonance are unable to respond significantly to the field inversions. In a strict three-state model, these molecules would nearly all become trapped in the high-mobility forward conformation under a typical FIGE switching regime. In a more realistic model, with a larger number of intermediates, they would accumulate in conformations intermediate between the fully forward state and the zero-mobility intermediate. In either case, they would be expected, as is observed, to have much higher mobility than the resonant molecules.

This discussion has deliberately not adopted any detailed view of the wedged conformation. The wedge is simply intended to symbolize the directionality of DNA conformations during steady-state electrophoresis. Molecular directionality is un-

doubtedly a property of all macromolecules undergoing steady-state electrophoresis under conditions such that the molecules are much longer than the effective sizes of the gel pores. Experiments employing intermittent unidirectional fields also provide evidence for conformational directionality[12,13]. Recent computer simulations of the chain dynamics of electrophoresing DNA molecules may provide some insight into the nature of the wedges[14]. These simulations suggest that, on a time average, the leading edge of the chain is relatively extended and under tension while tailing segments are increasingly likely to be hooked around obstacles in the gel. On the whole, these simulations are consistent with our earlier supposition that "the farther a segment is from the leading end of the molecule, the more likely it is to be penetrating the gel matrix along a path that is counterproductive to overall translocation of the molecule, and the larger the radius from which such counterproductive paths can be selected"[4]. However, the simulations emphasize that the concept of a wedge is, at best, a time-averaged description and that wedge collapse occurs relatively frequently[14].

This view of FIGE is readily extended to the more complex TFAGE geometry. Most particularly, it provides a simple explanation for the requirement that the transverse field in TFAGE intersect at an obtuse angle. This point is developed in Fig. 2 by comparing the expected behavior of rod-shaped (non-directional) and wedge-shaped (directional) conformations under TFAGE with field-intersection angles of 120 and 60°. The same considerations apply for any pair of angles chosen symmetrically about 90°. The key point is that a rod-shaped molecule can become realigned with the electric field after either a 60 or a 120° change in field direction simply by undergoing a 60° reorientation. To achieve this result, the molecule keeps the same leading segment in the 60° case, while it changes leading segments in the 120° case. Since, by definition,

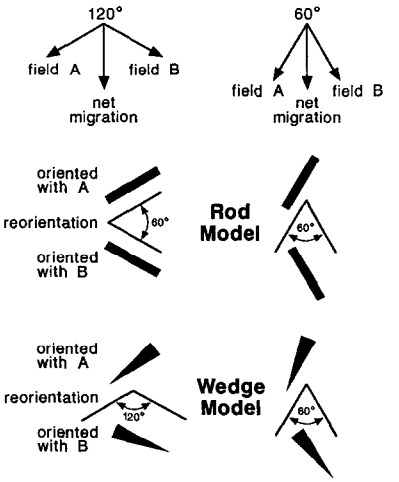

Fig. 2. Transverse-field-alternation gel electrophoresis (TFAGE). This diagram shows the geometric factors that apply when either rod- or wedge-shaped molecules become realigned with electric fields whose directions have been changed by 120 of 60°. The key point is that rod-shaped molecules can become realigned in either case with a 60° reorientation, while wedge-shaped molecules cannot. The particularly important case of the realignment of a wedge-shaped molecule following a 120° change in field direction is discussed in the text.

the two ends of a rod are considered equivalent, a rod-shaped molecule should be able to respond with equal ease to 120 and 60° changes in field direction.

In contrast, if the molecules are taken to be wedge-shaped, the nature of the molecular reorientation required when the field direction changes by 120° is fundamentally different from that required by a 60° change. If a wedge-shaped molecule is to keep the same leading segment, then it must undergo the full 120° reorientation. More plausibly, such molecules would be expected to undergo an end-to-end conformational change, as in FIGE, while also becoming reoriented by 60°. Since 60° reorientation, by itself, gives rise to size-independent migration, a reasonable hypothesis is that most of the size-dependence in TFAGE comes from essentially the same end-to-end conformational change that is central to the FIGE effect[4].

However, there is an important difference between the molecular motions involved in the response to a 120° TFAGE switching event, as opposed to a field-inversion event. This difference arises because a molecule that is undergoing an end-to-end conformational change in TFAGE is initially out of alignment with the electric field by 60°. Consequentiy, at the same time that the wedge is inverting, there is a large cross section for motions in the new field direction by local segments along the wedge. Most of these motions will be counterproductive with respect to overall molecular translocation. As a new path through the gel develops, these motions will give rise to added friction because many local segments will be hooked around components of the gel matrix that are obstacles with respect to the new path. The number of such segments will increase with the size of the molecules; consequently, the effect of this initial misalignment of an inverting wedge would be to introduce a drag on mobility that increases with molecular lengths and is uniquely associated with TFAGE rather than FIGE. This additional penalty for being large is likely to explain the absence in TFAGE of the dramatic double-valuedness of the size–mobility curve that is a prominent feature of FIGE.

CONCLUSION AND FUTURE PROSPECTS

Although heuristic and lacking in molecular detail, this view of pulsed-field phenomenology has the virtue of providing a unified perspective towards the diverse forms of PFGE. In particular, the following key points are accomodated:

(1) Large DNA molecules develop a high-mobility conformation during steady-state electrophoresis, which relaxes with time once the field is turned off[12,13]. This conformation is presumably the wedge itself, which although highly dynamic may represent an adequate description of the time-averaged structure[14].

(2) FIGE displays spectacularly size-dependent mobilities, even with equal forward and reverse field strengths, a result that is not predicted by any standard reptation theory[10,14].

(3) In contrast to the expectations of simple corner-turning models, 90° is an ineffective angle for TFAGE separations. Obtuse angles, which require the same type of wedge inversion featured in FIGE, are required.

(4) Size is a dramatically double-valued function of mobility in FIGE, while it is a largely single-valued function in TFAGE. Detailed consideration of the constraints on wedge inversion suggests that there is an additional penalty for being large in TFAGE that does not apply in FIGE.

Future progress in understanding the molecular basis of PFGE is likely to come from several directions. Biophysical studies of electrophoresing molecules should provide direct evidence of molecular conformations[15]. More advanced molecular dynamic simulations show promise of avoiding the oversimplifications inherent in formal statistical-mechanical theories[14]. In practical terms, it is possible to develop kinetic models of the type suggested in Fig. 1 into parameterized equations that relate mobilities to size and switching intervals, much in the way that enzyme kinetic measurements are treated. Finally, there is a great need for more systematic experimentation. The problem of developing pulsed-field instrumentation has been adequately solved in the context of present knowledge of electrophoretic behavior. Further advances depend on refining our knowledge of that behavior by a fruitful combination of theory and experiment.

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