Orientation of the agarose gel matrix in pulsed electric fields

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ABSTRACT

The technique of transient electric birefringence was used to investigate the effect of pulsed electric fields on the orientation of the agarose gel matrix. Orientation of the gel was observed at all electric field strengths. Very slow, time-dependent effects were observed when pulses of 10-100 V/cm were applied to 1% gels for 0.5-2 seconds, indicating that domains of the matrix were being oriented by the electric field. The sign of the birefringence reversed when the direction of the applied electric field was reversed, indicating that the domains tend to orient in the perpendicular direction after field reversal. Theories of gel electrophoresis will need to incorporate the orientation of the matrix in order to provide a complete explanation of electrophoresis in agarose gels.

INTRODUCTION

The mechanism responsible for separating very large DNA molecules by molecular weight during pulsed gel electrophoresis is not well understood, although various theories have been proposed (1-8). The migration of small DNA molecules in agarose gels at very low electric field strengths can be described by the Ogston theory of pore size distribution (9). The migration of larger molecules is better described by theories for reptation, the snake-like movement of elongated DNA molecules through the matrix (10,11). All theories of gel electrophoresis assume that the matrix itself does not orient in the electric field. This assumption has now been tested for agarose gels by studying the transient electric birefringence of the agarose gel matrix in pulsed electric fields.

The agarose matrix exhibits a strong electric birefringence signal at all gel concentrations and all electric field strengths. Short (msec) high voltage pulses appear to orient individual agarose chains or bundles of chains, or dangling ends of the matrix, giving rise to small positive birefringence signals with rapid rise and decay times. However, longer pulses typical of those used in pulsed field electrophoresis experiments (~10 V/cm, applied to

the gel for 0.5-2 sec) cause very slow time dependent changes in the agarose matrix, suggesting that domains in the gel are being oriented by the electric field. The sign of the birefringence, and hence the direction of orientation of the domains, can be reversed by reversing the direction of the applied electric field. The rapid orientation and reorientation of microdomains of the matrix in alternating pulsed electric fields would increase the fluidity of the matrix, making it easier for very large DNA molecules to migrate through the gel. Macroscopic orientation of the matrix also decreases the mobility of DNA restriction fragments in agarose gels.

MATERIALS AND METHODS

Samples. The agarose used for all birefringence measurements was Seakem LE agarose, lot no. 20105A, purchased from Bethesda Research Laboratories as "electrophoresis grade agarose". Gels containing 1% agarose were prepared by dissolving the agarose in boiling deionized water and pipeting the warm gel mixture into the space between a pair of birefringence electrodes which had been sealed on one side with a teflon plate (12,13). A second teflon plate was then placed on top of the electrodes. The gel mixture solidified within one minute. After removal of the teflon plates, the electrode assembly was placed in a birefringence cell containing distilled water. Equivalent results were obtained if the agarose was dissolved in 1 mM Tris buffer and the cell filled with the same buffer.

Electrophoresis. Electrophoresis gels containing 1% agarose were prepared from Seakem LE agarose (FMC), lot no. 61426. Gels were cast and run in TAE buffer (0.04 M Tris base plus 1 mM EDTA, brought to pH 8.0 with glacial acetic acid), using procedures described previously (9,14). All gels contained 2.5 $\mu\text{g/ml}$ ethidium bromide. Individually prepared gels were run in parallel lanes by using plexiglass spacers.

Electric birefringence. Electric birefringence measurements were made as described (13), using an apparatus which has been described briefly (12). The Kerr cell was a 1.00-cm quartz spectrophotometer cell, chosen for its negligible strain birefringence. The electrodes were parallel platinum plates with a 2.0-mm electrode separation, mounted on a lexan support of standard design (15). The position of the laser beam was adjusted to minimize the amount of scattered light from the gel; the stray light constant was usually about 5×10^{-5} with the gel in the light path. The temperature of all measurements was 20 °C; the calculated temperature rise during any one pulse was usually less than 0.1°C.

Electric field pulses ranging from 0.5-10 kV/cm in amplitude and 10 μ s to 3 ms in duration were generated by Cober high-power pulse amplifiers, Model 605P. Low voltage pulses ranging from 10-100 V/cm in amplitude and 0.1-2 sec in duration were generated by a newly designed pulser (Craig Fastenow, University of Iowa, Department of Biomedical Engineering). The rise and decay times of all pulses were 1 μ s or less.

The electric birefringence experiment is described in several reviews (15-17); see also 18). Briefly, if a macromolecule (or a gel matrix) is polar and asymmetric, it will interact with an applied electric field and tend to orient in the field. If the refractive index of the orienting material is different from that of the solvent, the refractive index will become different in directions parallel and perpendicular to the applied electric field. The difference in refractive index, called the electric birefringence, is thus a direct measure of orientation. At low electric field strengths the amplitude of the birefringence is proportional to the square of the electric field strength, E , as shown in Eq. (1).

$$\Delta n = n_{\parallel} - n_{\perp} = K n E^2 \quad (1)$$

Here, Δn is the birefringence, n is the mean refractive index of the solution, and K is a proportionality constant, known as the Kerr constant. Eq. (1) is known as the Kerr law (15).

The field-free decay of the birefringence after removal of the electric field is due to the randomization of the orientation of the birefringent particle because of Brownian motion. For an assembly of monodisperse rigid particles, the decay of the birefringence is given by

$$\Delta n = \Delta n_0 e^{-t/\tau} \quad (2)$$

where Δn is the birefringence at any time t , Δn_0 is the birefringence at the moment the field is removed, and τ is the relaxation time, which is related to the rotational diffusion constant, Θ , as shown:

$$\tau = 1/6\Theta \quad (3)$$

The rotational diffusion constant can be related to particle dimensions by standard equations (15-17).

RESULTS AND DISCUSSION

If the electric fields applied to the agarose gel were typical of those used in electric birefringence experiments, 1-10 kV/cm in amplitude and 10-1000 μ s in duration, the sign of the birefringence of the agarose matrix was positive, as shown in the inset of Fig. 1. The amplitude of the birefringence increased with the square of the electric field strength, as shown in Fig. 1, indicating that the Kerr law was obeyed. The rise and decay of the birefringence were characterized by time constants ranging from 10-200 μ s, depending on the length of the orienting pulse (13). If the electric

Fig. 1. Log-log plot of the amplitude of the birefringence of a 1% agarose gel, δ , as a function of the electric field strength, E . The pulse length was 250 μ s at all voltages. All amplitudes were corrected for the birefringence of water. The slope of the drawn line is 2.0 at $E \leq 5$ kV/cm. Inset: typical birefringence signal observed at $E = 2.8$ kV/cm. The horizontal scale is indicated. The direction of positive birefringence is up.

field was rapidly reversed in polarity, the birefringence decreased rapidly to about half its steady state value, and then increased to the same equilibrium value in the new field direction within about $60 \mu\text{s}$ (13). These results are consistent with the orientation of individual chains or bundles of agarose, or dangling ends of the matrix, parallel to the electric field lines.

Fig. 2. Oscilloscope traces of the birefringence signals exhibited by a 1.0% agarose gel at $E = 100 \text{ V/cm}$. (a), orientation during and immediately after the pulse; b), slower sweep, showing the complete decay of the birefringence; c), signal observed after application of two 500 ms pulses separated by 1 sec; d) 0.1% agarose solution. The rise and decay of the pulse are indicated by dashed lines. The pulse was applied for 500 ms in all cases. The vertical scale is arbitrary; the horizontal scale is indicated on each trace.

When smaller electric fields of much longer duration, typical of those used in pulsed field electrophoresis experiments, were applied to the agarose gels, complicated orientation patterns were observed. Typical electric birefringence signals observed when a pulse of 100 V/cm was applied to the gel for 500 ms are shown in Fig. 2. The birefringence signal, after an initial delay short on the time scale illustrated, became positive or negative during the pulse, often changed sign immediately after the pulse was removed, and

Fig. 3. Dependence of the birefringence signals observed for 1% agarose gels on electric field strength. (a,b) $E = 16$ V/cm, pulse length = 500 ms; (c,d), $E = 50$ V/cm, pulse length = 1 sec. Two sweep speeds are given for each voltage to illustrate the orientation during the pulse and the complete decay. The vertical scale is arbitrary; the direction of positive birefringence is up. The horizontal scale is indicated on each trace.

was followed by a much larger signal which passed through an extremum after several seconds and then decayed to zero. If a second pulse was applied to the gel before the birefringence had decayed to zero, further orientation of the matrix was observed, as shown in Fig. 2c. These results can only be explained by the orientation of domains or large clusters of the matrix by the longer pulses used at these lower electric field strengths.

Fig. 4. Oscilloscope traces of birefringence signals observed with fresh, independently prepared 1% agarose gels, oriented by the first pulse applied to the gel. (a,b), $E = 10$ V/cm, pulse length = 2 sec; (c,d), $E = 50$ V/cm, pulse length = 500 ms. In each case the sign of the birefringence of the gel remained constant when pulsed repeatedly at the given voltage or any other voltage. The vertical scale is arbitrary; the direction of positive birefringence is up. The horizontal scale is indicated on each trace.

The birefringence signals gradually became less complicated during and immediately after the pulse if the electric field strength was decreased from 100 V/cm to 10 V/cm, the voltage commonly used for pulsed electric field experiments, as shown in Fig. 3. At the lower voltages, the birefringence signal did not begin to decrease immediately after removal of the electric field, but continued to increase for several seconds before passing through an extremum and decaying to zero. These results suggest that partially oriented domains, once set in motion, continue to orient in the initial field

Fig. 5. Reversal of birefringence signal observed when the direction of the applied field is reversed. (a,b), $E = 10$ V/cm, pulse length = 2 sec. (c,d), $E = 50$ V/cm, pulse length = 500 ms. The vertical scale is arbitrary; the direction of positive birefringence is up. The horizontal scale is indicated on each trace.

direction even after the orienting force is removed. Similar results have been observed for relatively small, partially oriented DNA molecules in rapidly reversing electric fields (13).

The amplitude and overall appearance of the birefringence signal were independent of electric field strength if the product of amplitude and time (i.e., Vs) was constant. Therefore, orientation of the domains of the matrix was not due to heating and expansion of the gel, which would increase as E^2 . Slow time dependent effects attributed to expansion have been observed by linear dichroism (19), but the pulses used were 5-10 times longer than used in the present work.

The sign of the birefringence observed for each individually prepared gel was randomly positive or negative, as shown in Fig. 4. Thirty-six separate gels were prepared, with two-thirds exhibiting positive birefringence when the first pulse was applied to the gel. These results suggest that the direction of orientation of individual clusters or domains of the matrix could be either parallel or perpendicular to the electric field, depending on the microstructure of the individual gel. It is possible that the agarose domains are only slightly anisometric, and their direction of orientation is determined by whether they are slightly prolate or slightly oblate in shape.

The sign of the birefringence of each gel remained constant upon repeated pulsing, independent of the pulse length or applied electric field strength. However, the sign of the birefringence of a given gel could be reversed from positive to negative, or *vice versa*, by reversing the direction of the applied electric field, giving a signal that was the approximate mirror image of the original signal, as shown in Fig. 5. Similar results have been observed at lower field strengths by Sturm and Weill (20). The change in sign of the birefringence upon reversal of the electric field indicates that the domains of the agarose gel matrix, once oriented in the electric field, tend to orient in the perpendicular direction upon field reversal. Continuously reversing electric fields would keep such domains continuously reorienting parallel and perpendicular to the electric field, giving fluidity to the matrix and making it easier for large DNA molecules to migrate under the influence of the electric field.

Quasielastic light scattering studies have shown that 1% agarose gels contain scattering domains about 4μ in diameter (21). Perrin's equation for the rotational reorientation of a sphere, Eq. (4), can be used to estimate the birefringence relaxation time expected for the reorientation of a sphere of this size:

$$\tau = 4 \pi \eta a^3 / 3 kT \quad (4)$$

Here, a is the radius of the sphere, η is the viscosity of the solvent, k is Boltzmann's constant and T is the absolute temperature. From Eq. (4), the birefringence relaxation time expected for a sphere 4μ in diameter is calculated to be 8.3 sec. The relaxation time of the agarose matrix, *i.e.*, the time required for the birefringence to decay to $1/e$ of the maximum observed after removal of the electric field in Fig. 4 or 5, is 12 sec; the relaxation time estimated from the tangent to the initial decay (15) of this peak is 9.5 sec. These values are of the same order of magnitude as the relaxation time estimated from the size of the domains observed by quasielastic light scattering, suggesting that domains of this size are indeed orienting in the electric field.

Similar birefringence signals were observed for 0.1% solutions of LE agarose, which do not gel, except that the sign of the birefringence was always positive when the first pulse was applied to the solution (Fig. 1d). The amplitude of the birefringence increased upon repeated pulsing, indicating that the chains were aggregating in the electric field (not shown). The sign of the birefringence could be reversed from positive to negative by reversing the direction of the applied electric field, indicating that the same type of orientation behavior observed for agarose gels was occurring in agarose solutions.

The orientation of the agarose matrix in an electric field can also affect the mobility of DNA restriction fragments during gel electrophoresis. One section of a 1% agarose gel was cast in the usual way and allowed to cure overnight, after which a second portion of the gel was poured in an adjacent lane and allowed to solidify while an electric field of 2.6 V/cm was applied perpendicular to the eventual direction of the electrophoresis. About two hours after the second portion of the gel solidified, DNA samples were applied to the gel and electrophoresed with $E = 2.6$ V/cm. The DNA fragments in the "perpendicularly oriented" gel migrated about 7% slower than identical fragments in the control gel, presumably because of macroscopic orientation of the matrix during gel formation. The oriented matrix lost its "perpendicular" orientation after 24 hours, because DNA fragments in both portions of the gel migrated with the same velocity the following day. Therefore, the matrix must undergo a randomization of the oriented domains with time, probably because of rearrangement of the hydrogen bonds holding the agarose chains in their three dimensional array (22).

CONCLUDING REMARKS

The results presented here have demonstrated that the agarose gel matrix can be oriented by electric fields of the amplitude and duration usually used in pulsed gel electrophoresis experiments. The orientation of the matrix can be observed both in electric birefringence experiments and in electrophoresis experiments in a constant electric field. The matrix appears to orient in domains several microns in size. These domains may be slightly prolate or oblate in shape, giving rise to birefringence signals which are randomly positive or negative in sign. The domains appear to change their orientation, from parallel to perpendicular, or *vice versa*, when the electric field is reversed in polarity. Constant orientation and reorientation of the matrix in pulsed electric fields would give fluidity to the matrix, making it easier for very large DNA molecules to migrate in response to the electric field gradient. Theoretical treatments of DNA electrophoresis in agarose gels will need to include the orientation of the matrix to provide a complete explanation of the phenomenon.

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