

Restriction Enzyme Kinetics Monitored by UV Linear Dichroism[†]Matthew R. Hicks,^{*,†,§} Alison Rodger,[‡] Christopher M. Thomas,[§] Sarah M. Batt,[§] and Timothy R. Dafforn[§]*Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K., and Department of Biological Sciences, University of Birmingham, Birmingham B15 2TT, U.K.**Received January 26, 2006; Revised Manuscript Received April 24, 2006*

ABSTRACT: The use of linear dichroism (LD) spectroscopy for biological applications has been brought to the forefront recently by our development of thermostated microvolume Couette cells. We present a method for following the digestion of DNA by restriction endonucleases in real time without the use of any extrinsic dyes or labels. This is accomplished using linear dichroism spectroscopy (the differential absorbance of light polarized parallel and perpendicular to the sample orientation axis). The differential absorbance signal depends on the degree of alignment of the molecules. In this case the DNA is aligned by Couette flow (flowing the solution in the annular gap between two concentric cylinders), and we monitor the increase in alignment upon linearization of a circular DNA molecule. In addition, we observe a decrease in alignment upon further digestion and subsequent shortening of the DNA. Ten enzymes were investigated: seven enzymes with a single cut site (*EcoRI*, *KpnI*, *NdeI*, *NotI*, *NruI*, *SmaI*, *XbaI*), two enzymes with two cut sites (*BstZ17I*, *EagI*), and one enzyme with no cut site (*ClaI*). LD, as implemented in this new assay, is broadly applicable across a wide range of DNA-modifying enzymes and compounds and, as such, is a useful addition to the toolbox of biological characterization.

The type II restriction endonucleases are a family of enzymes that recognize specific DNA sequences and cleave at fixed positions at or close to that sequence to produce 5'-phosphates and 3'-hydroxyls. They may act as monomers, dimers, or tetramers (1). Restriction endonucleases evolved to protect prokaryotic organisms from invasion by foreign DNA; their own DNA is protected by specific DNA methylation patterns (N4 or C5 methylation of cytosine or N6 methylation of adenine) whereas the foreign DNA is not. The foreign DNA is therefore susceptible to cleavage (restriction) by the host enzyme (2). The thousands of restriction enzymes that have been identified to date are also extremely important tools for the molecular biologist. They are used in many techniques that require DNA manipulation and have applications in, for example, diagnostics and recombinant protein production (3).

To detect and characterize the cleavage of DNA (and thus also monitor the activity of the enzyme), one can use a variety of techniques, the most common of which is to separate the DNA fragments, postincubation, according to molecular size using electrophoresis, usually agarose gel electrophoresis. For time-based measurements each time point requires one lane on a gel, limiting the number of points that can be practically measured. It is more desirable to be able to follow the reaction in real time. There have been a number of approaches developed to follow the cleavage of DNA in real time. Many of these are fluorescence-based and

include fluorescence resonance energy transfer (FRET)¹ (4), dual-color fluorescence cross-correlation spectroscopy (5, 6), fluorescence anisotropy combined with FRET (7), or the use of a fluorophore and a quencher at the ends of a nucleic acid hairpin (8). Alternative approaches include the use of immobilized nucleic acids on evanescent wave sensors (9, 10) and ferrocene-modified oligonucleotides coupled with electrochemical detection (11). All of these approaches require modification of the substrate with either a label or a means of immobilization on a surface. One can measure restriction enzyme activity using an assay based on the hyperchromic effect; however, the substrate DNA (synthetic oligonucleotides) must be designed such that it is double stranded before cleavage and becomes single stranded after cleavage (12).

As an alternative to the methods that require DNA modification or the design of specific oligonucleotides, we proposed that linear dichroism (LD) could be used to monitor the cleavage of unmodified DNA in real time. LD is the difference in absorbance of light polarized parallel and perpendicular to the sample orientation axis. Chromophores in the sample (the DNA bases in this case) absorb the two polarizations of light to different extents depending on their

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* Corresponding author. Telephone: +44 (0)2476 523293. Fax: +44 (0)2476 524112. E-mail: Matthew.Hicks@warwick.ac.uk.

[‡] University of Warwick.

[§] University of Birmingham.

¹ Abbreviations: A, absorbance; A_{||}, absorbance of light polarized parallel to the sample orientation; A_⊥, absorbance of light polarized perpendicular to the sample orientation; α_i, angle the transition moment makes with the axis of alignment; bp, base pairs; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; HT, high tension (voltage); i.d., inside diameter; K_M, Michaelis constant; LB, Luria-Bertani; LD, linear dichroism; LD₂₆₀, linear dichroism signal at 260 nm; LD^r, reduced linear dichroism signal; o.d., outside diameter; PMT, photomultiplier tube; S, the orientation parameter of the sample; [S], substrate concentration; t_{1/2}, half-time for restriction of substrate DNA; TAE, Tris-acetate-EDTA buffer; V, velocity of enzyme reaction; V_{max}, maximum velocity of enzyme reaction.

orientation in the sample. The magnitude of the linear dichroic signal for a chosen transition is given by the equation

$$LD^r = \frac{LD}{A} = \frac{A_{\parallel} - A_{\perp}}{A} = \frac{3S}{2}(3 \cos^2 \alpha_i - 1) \quad (1)$$

where LD^r is the reduced LD (LD divided by the total absorbance of the sample, A), A_{\parallel} is the absorbance of light polarized parallel to the sample orientation, and A_{\perp} is the absorbance of light polarized perpendicular to the sample orientation. The angle the i th transition moment makes with the axis of alignment is denoted by α_i , and S is the orientation parameter of the sample: it equals 1 for perfect alignment and 0 for an isotropic sample. See Rodger and Nordén (13) for a more comprehensive review of dichroic spectroscopy.

The extent to which a molecule is oriented by shear flow (its S value) depends on several factors including flow rate, viscosity of the solvent, rigidity/flexibility of the molecule, and the ratio of the long and short axes of the molecule. The LD signal is thus expected to change if the extent of orientation (S) and/or the average base orientation (α_i) changes. We hypothesized that the magnitude of the LD signal would change when supercoiled DNA was linearized and also when the initial product was further cleaved. The change in the LD signal, caused by the changes in S and α_i upon cleavage of the DNA, could thus be used as an assay to follow the enzyme reaction in real time without the need for any dyes or labels. The precise structure of the plasmid is unclear; however, because of the relatively compact nature of circular supercoiled molecules and the nonuniform direction of the bases, it would be reasonable to expect the supercoiled plasmid to give a smaller LD signal than the linearized DNA molecule. Further cleavage of the DNA (if there are multiple restriction sites) will result in shorter linear DNA molecules whose orientation in the flow is expected to be less than that of the longer DNA, resulting in a reduction in measured LD. To investigate this, we monitored the LD signal throughout reactions of DNA with different restriction enzymes.

Here we present some examples of the way in which this potentially generally applicable technique could be used. Seven restriction enzymes were tested for cleavage at their chosen single site of the substrate DNA; an enzyme that does not cut was also tested. Two additional enzymes that cut at two sites each were tested to determine if the two kinetic events could be followed.

The data were analyzed by calculating Michaelis–Menten kinetic parameters from the LD data; an example of this is given for *EcoRI* using the initial rate method. The kinetics of the enzymes that cut twice are more complicated than the single cut ones. We are working on characterizing these reactions by analyzing the complete reaction progress curves numerically in terms of different kinetic models. However, this sort of in-depth analysis is beyond the scope of the present study and will be the subject of subsequent work.

EXPERIMENTAL PROCEDURES

DNA Purification. The plasmid used in this work was a pCDNA3.1 derivative (Invitrogen, Paisley, U.K.) and was 6882 base pairs (bp) in length. XL10 Gold *Escherichia coli* cells (Stratagene, La Jolla, CA) were transformed with plasmid DNA and grown overnight at 37 °C on Luria–

Bertani (LB) agar supplemented with 100 $\mu\text{g}/\text{mL}$ (270 μM) sodium salt of ampicillin (Sigma-Aldrich Co. Ltd., Gillingham, U.K.). A single colony was transferred to LB broth and grown overnight at 37 °C. The DNA was purified from the *E. coli* cell pellets using a Concert Maxi-Prep system (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions and dissolved in ultrapure water (resistivity 18.2 $\text{M}\Omega\cdot\text{cm}$). The DNA was stored frozen at -20 °C in small aliquots until required.

Gel Electrophoresis and Restriction Enzyme Reactions. Electrophoresis was performed in 0.7% (w/v) agarose gels in $1\times$ Tris–acetate–EDTA (TAE) buffer (Fisher Scientific, Loughborough, U.K.) (3). All samples were run in $1\times$ DNA loading buffer (Bioline, London, U.K.) (3). Samples containing 40 ng of DNA from the end point of the restriction enzyme kinetic experiments were run on agarose gels to check for digestion. The enzymes were supplied by the following companies: *ClaI* and *NruI* (Invitrogen Ltd., Paisley, U.K.); *BstZ17I*, *EagI*, *EcoRI*, *KpnI*, *NdeI*, *SmaI*, and *XbaI* (New England Biolabs U.K., Hitchin, U.K.); *NotI* (Promega, Southampton, U.K.). The cut sites of the enzymes are as follows with the recognition sequence followed by the location of that sequence in our plasmid; the caret indicates the position in the recognition sequence at which the enzyme cuts: *BstZ17I* (GTA \wedge TAC: 1831, 4694), *ClaI* (AT \wedge CGAT: no site), *EagI* (C \wedge GGCCG: 933, 3630), *EcoRI* (G \wedge AAATTC: 2415), *KpnI* (GGTAC \wedge C: 2450), *NdeI* (CA \wedge TATG: 489), *NotI* (GC \wedge GGCCGC: 934), *NruI* (TCG \wedge CGA: 212), *SmaI* (CCC \wedge GGG: 3535), and *XbaI* (T \wedge CTAGA: 921).

For production of linear DNA (used to assess LD signal as a function of rotation speed and LD signal as a function of concentration), plasmid DNA (10 μg) was digested at 37 °C for 2 h with 50 units of *EcoRI* (2.5 μL) in the buffer supplied with the enzyme (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl_2 , 0.025% Triton X-100, pH 7.5) in a total volume of 50 μL . Size markers were kilobase DNA markers (Amersham Pharmacia Biotech, Chalfont St. Giles, U.K.). Buffers for other enzymes were as supplied and as follows: *ClaI* (50 mM Tris-HCl, 10 mM MgCl_2 , pH 8.0), *NruI* (50 mM Tris-HCl, 10 mM MgCl_2 , 50 mM NaCl, 50 mM KCl, pH 8.0), *BstZ17I* (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl_2 , 1 mM DTT, pH 7.9), *EagI* (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl_2 , 1 mM DTT, pH 7.9), *EcoRI* (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl_2 , 0.025% Triton X-100, pH 7.5), *KpnI* (10 mM Bis-Tris propane HCl, 10 mM MgCl_2 , 1 mM DTT, pH 7.0), *NdeI* (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9), *SmaI* (20 mM Tris–acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9), *XbaI* (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl_2 , 1 mM DTT, pH 7.9), *NotI* (6 mM Tris-HCl, 150 mM NaCl, 6 mM MgCl_2 , 1 mM DTT, pH 7.9).

Linear Dichroism. Linear dichroism (LD) was measured on a Biologic MOS-450 spectropolarimeter adapted for LD spectroscopy. The instrument was purged with nitrogen at a flow rate of 10 L/min. The bandwidth was 2 nm, and sample volumes were 50 μL for all measurements. The thermostated, microvolume Couette cell was manufactured by Crystal Precision Optics (Rugby, U.K.) and is available via Kromatek (Great Dunmow, U.K.). A schematic of the cell is shown in Figure 1. Reactions were initiated by mixing the enzyme

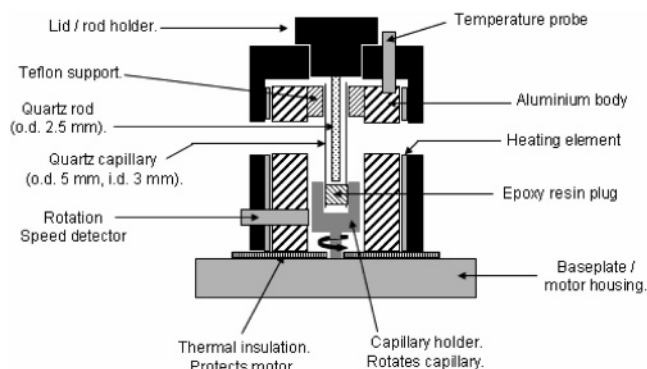


FIGURE 1: Schematic diagram of the microvolume, thermostated Couette cell (not to scale).

with the other reactants immediately prior to LD measurement. This results in a “dead time” of 10 s.

Wavelength scanning spectra were measured between 220 and 320 nm with a data interval of 1 nm and a data averaging time of 0.2 s at each data point; four spectra were averaged. Baseline spectra were subtracted from the raw data and were measured with the sample in the cell but with the rotation voltage set at 0 V (the capillaries are optically uniform and give the same baseline in all positions). Kinetic traces were measured at 260 nm, and the photomultiplier high-tension voltage (HT) was fixed at a value which gave an initial PMT output voltage of 5 V (dynamic range is 0–10 V). This was typically around 200 V, depending on sample concentration. The cell temperature was maintained at 37 ± 0.2 °C. Applied voltage to the motor was maintained at the stated value ± 0.02 V.

Data Analysis. The initial rates for the enzyme kinetics were taken from a linear least-squares fit to the linear part of the LD_{260} versus time curve, typically between 200 and 1000 s. This was after temperature and flow equilibration and when the kinetics had reached steady state but before the substrate becomes limiting. The rates were converted from LD units to concentration units using the slope of a calibration curve of LD_{260} versus concentration of linearized plasmid DNA. The rates were fitted to the Michaelis–Menten equation (eq 2) using Kaleidograph (Synergy Software, Reading, PA):

$$V = V_{\max}[S]/(K_M + [S]) \quad (2)$$

where V is the velocity of the reaction, V_{\max} is the maximum velocity, $[S]$ is the substrate concentration, and K_M is the substrate concentration at which V is half-maximal ($V_{\max}/2$).

RESULTS

The purified 6882 bp supercoiled DNA plasmid was first linearized using the restriction endonuclease *EcoRI*, which cuts at the sequence G[^]AATTC (the caret indicates the position at which the phosphodiester bond is cleaved).

Signal versus Rotation and Concentration. The LD signal of linear DNA of 6882 bp varied with the speed of rotation of the quartz sample capillary. Spectra were measured over a range of voltages between 1 V (560 rpm) and 5 V (4800 rpm) with a 0.5 V increment (Figure 2A), and the LD signal at 260 nm was plotted against rotation speed (Figure 2B). The increase in the LD_{260} as a function of rotation speed is due to an increase in the alignment of the sample. It is clear

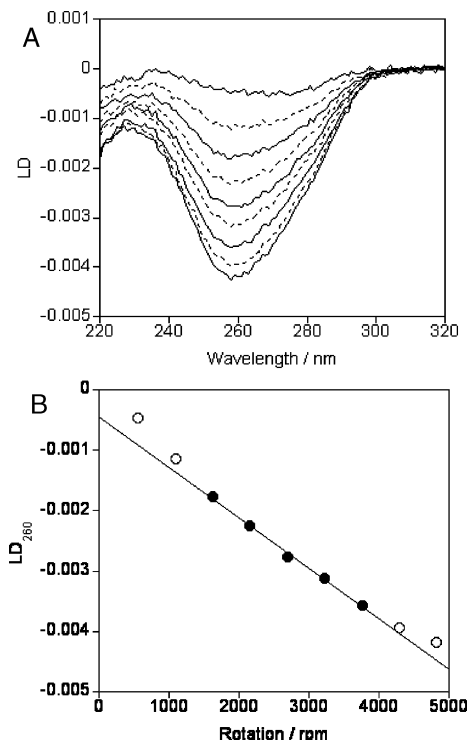


FIGURE 2: Linear dichroism of DNA at different rotation speeds. (A) LD spectra of 20 ng/ μ L DNA after linearization with *EcoRI*. The smallest signal is given by a rotation speed of 1 V (560 rpm), and spectra increase in magnitude with increasing voltage in 0.5 V increments up to 5 V (4800 rpm). (B) LD signal at 260 nm versus rotation voltage. The signal increases approximately linearly between 2 V (1600 rpm) and 4 V (3800 rpm).

that the signal approaches linearity with rotation speed in the middle of the range but deviates from linearity at the extremes, as discussed below. The largest signal obtained within the linear range was at 4 V (3800 rpm), and for this reason subsequent data are all collected at this rotation speed.

The LD signal should also be directly proportional to concentration unless the viscosity of the solutions changes. To test this, we measured spectra of different concentrations of linear DNA at a constant rotation speed of 3800 rpm (Figure 3A). The LD_{260} signal was plotted against concentration of DNA bases, and a linear relationship was observed over the range tested (Figure 3B). The slope of the fit was $-62.9/M$ of DNA bases ($-8.66 \times 10^5/M$ of linearized plasmid) with a good fit (R value of 0.99903). This was repeated for the supercoiled plasmid, and a value of $-20.3/M$ of DNA bases ($-2.79 \times 10^5/M$ of supercoiled plasmid) was obtained. The difference between these values is $-42.6/M$ of DNA bases ($-5.87 \times 10^5/M$ of plasmid that has been linearized), which provides a constant for conversion of the LD_{260} versus time plots that will be used for the kinetic analysis.

Kinetics of Restriction Endonuclease Reactions. The LD_{260} signal of DNA in the presence of the ten different restriction enzymes was measured over a period of 1 h. The LD signal as a function of time enables one to follow the effect of the restriction enzyme on the plasmid DNA. Three examples are shown in Figure 4. The enzyme *EcoRI* has a single recognition site in the plasmid and as such is expected to produce a linear DNA molecule. Figure 4A demonstrates that the observed kinetic trace shows an increase in negative LD_{260} . This is consistent with an increase in orientation with

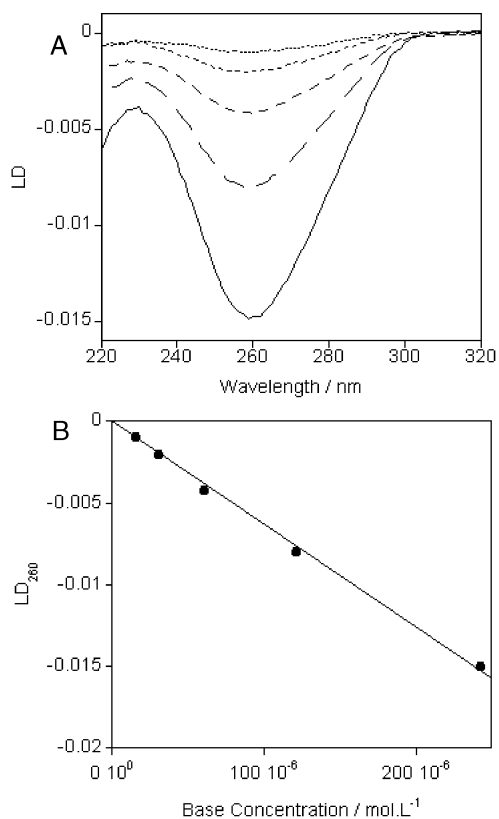


FIGURE 3: Linear dichroism of DNA at different concentrations. (A) LD spectra of linear DNA. Base concentrations were 15 μM (dotted line), 30 μM (short dashed line), 61 μM (dashed line), 121 μM (long dashed line), and 242 μM (solid line). (B) Data from (A) converted to the signal at 260 nm versus DNA concentration.

linearization of the plasmid, as predicted. This shows that LD can be used to follow restriction enzyme reactions in real time without the need for any dyes or labels.

The effect of enzymes with two cleavage sites in the plasmid DNA is also apparent in the LD traces of Figure 4B,C where the enzymes *EagI* and *BstZ17I* were used, respectively. In both cases the increase in the negative LD signal at earlier time shows the linearization of the plasmid. The decrease in the negative LD signal observed at later time reflects the second cleavage.

A further seven restriction enzymes were analyzed for their ability to cut the plasmid. Six of these enzymes had a single recognition site in the plasmid, and one (*ClaI*) had no site. We were initially surprised to find that the LD signal magnitudes at the end of some of the reactions were not as high as with *EcoRI* (Table 1), suggesting incomplete restriction of the sample. To verify by an established method the extent to which the enzymes cut, the samples were run on an agarose gel at the end of each LD kinetic run (Figure 5). It can be seen that *SmaI* (lane i) and *XbaI* (lane j) cut the DNA only partially. To a lesser extent this is also seen for *NdeI* (lane f). These gel results are thus entirely consistent with the LD results, and this confirms that the final LD signal can be used as a check for degree of digestion (most usually whether digestion is complete).

From the LD kinetic data, the time for half the substrate DNA to be cut ($t_{1/2}$) was calculated for each of the enzymes where this was appropriate, i.e., for those where efficient cutting at a single site occurred. The results are summarized in Table 1.

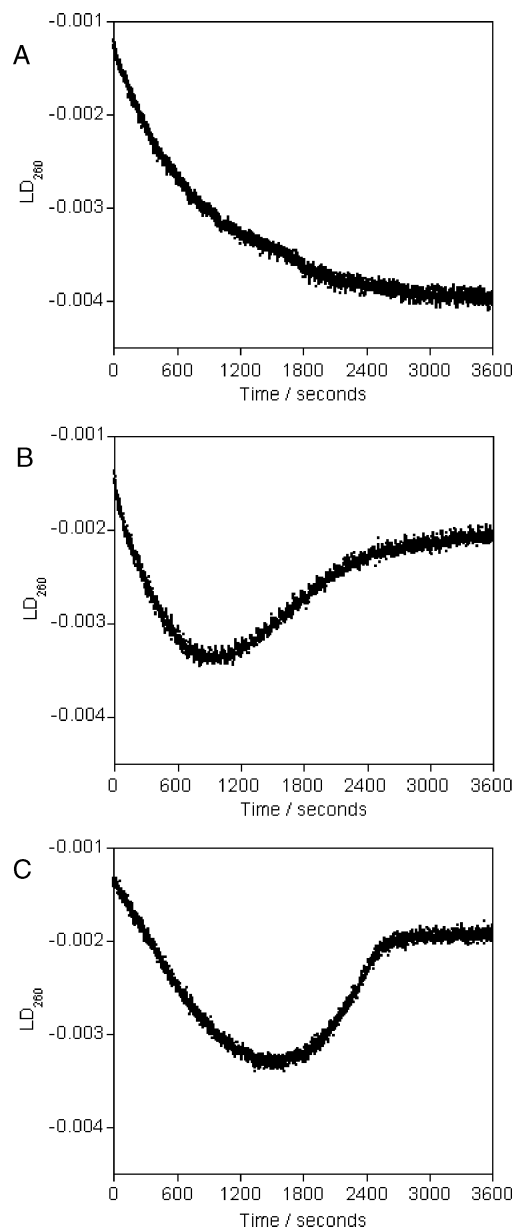


FIGURE 4: Kinetics of restriction digests of a circular, supercoiled plasmid. (A) Plasmid was digested with *EcoRI*, which has a single cut site in the DNA sequence. (B) Plasmid digestion with *EagI*, which has two cut sites in the DNA sequence. (C) Plasmid digestion with *BstZ17I*, which also has two cut sites in the DNA sequence.

Table 1: Restriction Digestion of Plasmid DNA by Restriction Enzymes with ≤ 1 Recognition Sites in the Plasmid DNA Sequence^a

enzyme	final LD ₂₆₀	cut	$t_{1/2}/\text{min}$
<i>ClaI</i>	-0.0010	none	N/A
<i>EcoRI</i>	-0.0040	complete	9.7
<i>KpnI</i>	-0.0039	complete	0.83
<i>NdeI</i>	-0.0038	near complete	0.47
<i>NotI</i>	-0.0041	complete	1.2
<i>NruI</i>	-0.0041	complete	0.83
<i>SmaI</i>	-0.0023	partial	N/A
<i>XbaI</i>	-0.0022	partial	N/A

^a The enzymes were at a concentration of 0.4 unit· μL^{-1} , and the DNA was at a concentration of 40 ng· μL^{-1} .

To determine the kinetic parameters K_M and V_{max} using the initial rates, the enzyme concentration should be significantly below K_M . An *EcoRI* concentration of 0.2 unit· μL^{-1} was used for the kinetic experiments summarized in Figure

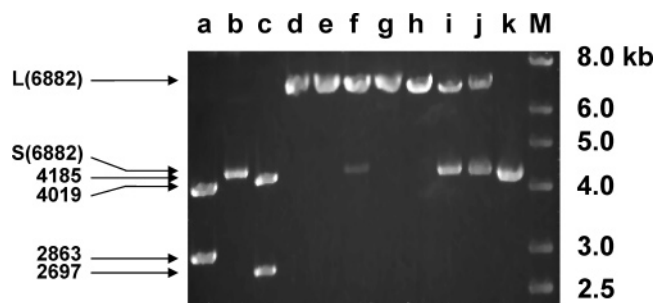


FIGURE 5: Agarose gel electrophoresis of restriction endonuclease reaction products: (a) *BstZ17I*, (b) *ClaI*, (c) *EagI*, (d) *EcoRI*, (e) *KpnI*, (f) *NdeI*, (g) *NotI*, (h) *NruI*, (i) *SmaI*, (j) *XbaI*, (k) uncut plasmid, and (M) molecular size markers. The arrows denote migration positions for the bands; linear is denoted by L and supercoiled by S.

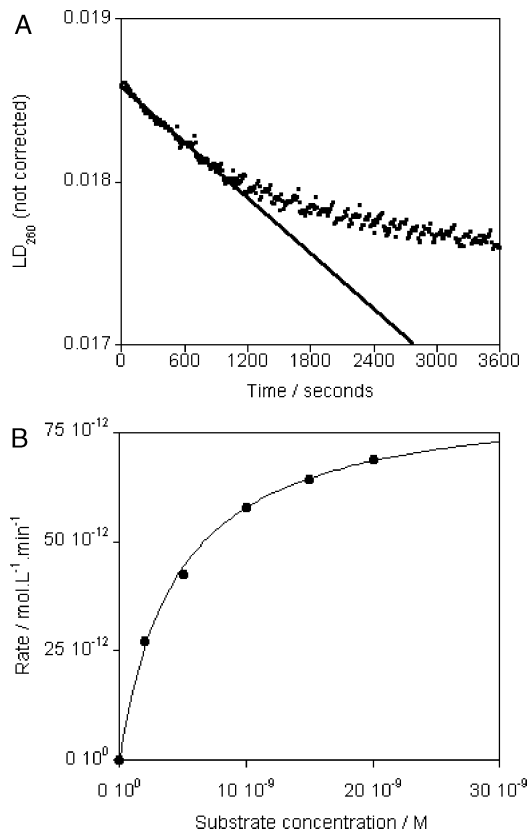


FIGURE 6: *EcoRI* restriction enzyme kinetics in detail. (A) The initial rate of product formation in the restriction digest was calculated from the slope of the steady-state part of the LD versus the time curve at different DNA (substrate) concentrations. This example is for 10 nM plasmid (138 μ M bases). The rate was converted to concentration per unit time using the slope of the curve in Figure 3B. (B) Initial rate versus substrate concentration. The kinetic parameters K_M and V_{max} can be calculated by fitting this rectangular hyperbolic curve to eq 2.

6. Using a specific activity of 6×10^6 units \cdot mg $^{-1}$ (the average of the range provided by the manufacturers) and a dimer molecular mass of 62 kDa, this corresponds to an enzyme concentration of 538 pM. Substrate (plasmid) concentration was varied from 2 to 20 nM. The initial rates were measured as illustrated in Figure 6A, taking the slope of the LD_{260} versus time curve over the steady-state part of the reaction. These rates were converted to concentration units and were plotted versus substrate concentration. The resulting rectangular hyperbola is described by the Michaelis–Menten equation (eq 2). By fitting to the data using

Kaleidograph (based on the Levenberg–Marquardt algorithm), the parameters V_{max} and K_M were found to be 84 pM \cdot min $^{-1}$ and 4.5 nM, respectively.

DISCUSSION

LD Signal versus Rotation and Concentration. Between 2 and 4 V the LD signal at 260 nm is linear with rotation voltage. The maximum sample alignment without inducing Taylor vortices and turbulent flow (14) is desired. At low rotation rates the rotation speed may not be stable, and at higher flow rates there may be turbulent flow. We therefore decided that the maximum voltage that should be applied must be within the linear response regime; this was 4 V in this case.

The LD signal of the linearized DNA plasmid of 6882 bp at constant rotation voltage varies linearly with concentration over at least the range 15–240 μ M bases. This corresponds to approximately 1–20 nM plasmid. The linear relationship facilitates a conversion of LD units to plasmid concentration, so that enzymatic rates may be calculated in units of concentration per unit time by dividing by a constant: $-42.6/M$ in this work. Note that the relationship of LD signal per unit concentration will depend on several factors, most notable DNA length, but also sequence (and hence flexibility), sample viscosity, and temperature; it should therefore be determined for the system being investigated.

Kinetics of DNA Cleavage by Enzymes with a Single Site in the Substrate Plasmid. We have demonstrated that one can follow the cleavage of plasmid DNA using LD spectroscopy to provide a real-time, continuous monitoring of the reaction. Some enzymes are known not to cut as efficiently as others. This is reflected in the magnitude of the LD signal at the end of the reaction (Table 1) and can be seen in Figure 6. Less efficient enzymes in this study include *SmaI*, *XbaI*, and to a minor extent *NdeI*. To confirm the LD signal correlated with cut efficiency, gels were run of the end point solutions.

To demonstrate that this method can be used quantitatively for determination of kinetic parameters, we undertook a more detailed study of the kinetics of one of the enzymes. We chose *EcoRI* because this is the most widely studied and used of the restriction enzymes, providing literature values for comparison. Our K_M value of 4.2 nM for *EcoRI* is in agreement with the literature values, which range between 1 and 10 nM for a range of plasmid substrates (15–18). The k_{cat} (or turnover number) equals $V_{max}/$ enzyme concentration. The enzyme concentration was determined using a specific activity value of 6×10^6 units \cdot mg $^{-1}$, which was the mean of the range ($2-10 \times 10^6$ units \cdot mg $^{-1}$) given by the manufacturers (New England Biolabs U.K., Hitchin, U.K.). From our experiments k_{cat} therefore equals 0.05–0.26 min $^{-1}$. Note that the error in the measured value is due to uncertainty in the enzyme concentration and not error in the experimental data or fitting. This is at the lower end of reported k_{cat} values. However, the turnover number is variable and depends on the reaction conditions and will be affected by the amount of active enzyme present. The amount of active enzyme in a stock will also diminish over time. It is also possible using the assay presented here to detect incomplete digestion of the DNA. This is apparent from a smaller LD signal than for complete digestion.

Kinetics of DNA Cleavage by EagI and BstZ17I. Cleavage of the substrate DNA at two separate sites was also followed using LD. The increase in the negative LD₂₆₀ signal that is observed in the first part of the curves in Figure 4B,C is due to the increased alignment that occurs upon linearization of the supercoiled plasmid. The subsequent decrease in the negative signal is due to the linear DNA molecule being cut a second time to give two shorter molecules. The shorter molecules align less well and thus give a smaller LD₂₆₀. The fragment sizes that result from DNA cleavage are 4185 bp + 2697 bp for *EagI* and 4019 bp + 2863 bp for *BstZ17I*. These fragment sizes are similar and should result in a similar LD₂₆₀ upon complete digestion. This is observed at the end of the kinetic traces with values within 5% of each other. The time dependency of the LD for *EagI* is much as one might expect, with an initial phase resembling the *EcoRI* curve and the gradually increasing effect of the second cleavage. The case for *BstZ17I*, which is consistently reproducible, is unusual because the second cleavage event proceeds with a faster rate than the first but only after the completion of the first cleavage. This suggests that the second site may have a fast rate constant for cleavage but that the binding of the enzyme to this site is weak. This may be because of the site being buried and/or the sequence context. Some restriction enzymes require two DNA binding sites to initiate cleavage, resulting in complex kinetics; see, for example, the work on *SfiI* from Halford's group (19, 20). Work is in progress to mathematically model the more complex kinetic processes and to work out the mechanistic implications. The conclusion for the purposes of this work is that LD not only monitors the restriction reaction but also easily highlights any unusual kinetic aspects to the enzyme's activity.

CONCLUSIONS

We have demonstrated the versatility and ease of use of linear dichroism for the study of enzyme-catalyzed reactions of useful DNA substrates. There are many possible applications of this technique: not least, the study of restriction enzyme kinetics under different reaction conditions, batch to batch testing for restriction enzyme manufacturers, restriction enzyme-based assays, and following reactions in real time for molecular biology applications.

One of the key advantages of LD is that it is nondestructive, and the sample can be recovered after measurement. Digestion can be monitored in real time and the sample removed for subsequent genetic manipulation as soon as cleavage is complete. This has the advantage of saving time and avoiding "overdigestion", which may result in star activity where nonspecific sites are cut by some enzymes (21).

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