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The kinetic properties of cruciform extrusion are determined by DNA base-sequence

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ABSTRACT

The extrusion kinetics of two cruciforms derived from unrelated DNA sequences differ markedly. Kinetic barriers exist for both reactions, necessitating elevated temperatures before extrusion proceeds at measureable speeds, but the dependence upon temperature and ionic strength is quite different for the two sequences. One, the ColE1 inverted repeat, exhibits a remarkably great temperature dependence of reaction rate and is suppressed by moderate amounts of NaCl or $MgCl_2$. In contrast, the other, a synthetic inverted repeat present in pIRbke8, shows more modest temperature dependence and has a requirement for the presence of salt, with optimal concentrations being 50 mM NaCl or 100 µM $MgCl_2$. Under optimal conditions, cruciform extrusion rates are fast (t1/2 <60m) at 37°C for both sequences at native superhelix densities. In 50 mM NaCl the pIRbke8 inverted repeat is characterised by an Arrhenius activation energy of 42.4 ±3.2 kcal $mole^{-1}$. The differences in kinetic properties between the two sequences indicate that DNA base sequence is itself an important factor in determining cruciform kinetics, and possibly even in the selection of the mechanistic pathway.

INTRODUCTION

Sequence-dependent DNA structural perturbations have generated considerable interest in recent times. Perhaps the most extreme example is the cruciform, involving a complete reorganisation of base-pairing and stacking from the regular inter-strand-bonded duplex to the intra-strand-bonded hairpin loop structures. Cruciform structures were first recognised as theoretical conformational possibilities when DNA sequence possessed two-fold symmetry as an inverted repeat (1,2). The experimental verification was only obtained once supercoiled DNA molecules were examined (3-5), since the unfavourable free energy of formation of the cruciform must be offset by the

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topological relaxation is the circular molecule. Cruciform structures have subsequently been demonstrated in natural and constructed DNA sequences, using a variety of techniques including enzymic and chemical probing and topological methods (6-15).

In order to understand the nature of cruciform structures, we need to describe their three dimensional structures and their physical chemistry. We have several studies of cruciform structure proceeding, including chemical probing of loop geometry (G.W.Gough, K.M.Sullivan and D.M.J.Lilley, in preparation) and studies of the geometry of the four-way junction at the base of the cruciform stems (16). The physical chemical description of cruciforms requires two kinds of information viz thermodynamic and kinetic. The thermodynamics of a number of cruciforms has been studied by examining cruciform extrusion as a function of the extent of negative supercoiling (10,13,17-21). In all cases there is excellent agreement that there is a narrow range of linking difference over which the cruciform becomes stable, in agreement with earlier theoretical predictions (22,23). The free energy of formation has been measured for three inverted repeats, with values of 18 kcal mole^{-1} for pUC7 (20), 17 kcal mole^{-1} for pAC103 (13) and 18 kcal mole^{-1} for ColE1 (21), each in their respective buffers.

The first kinetic studies of cruciform extrusion were performed by Mizuuchi et al (7), who showed that the reaction was surprisingly slow. Subsequent kinetic studies of several cruciforms (13,20,24,25) have confirmed that interconversion between regular duplex and the cruciform is not facile, and that there are considerable kinetic barriers which must be surmounted. In the present study I have made a comparison of the kinetic properties of two inverted repeats of unrelated sequence, one natural and one synthetic. The results show that the kinetics of cruciform extrusion for the two sequences differ markedly in their dependence upon temperature and ionic strength, indicating that base sequence may be a major determinant of kinetic properties.

EXPERIMENTAL

Plasmids

pColIR215 is a recombinant plasmid derived by cloning a EcoRI, Sau3AI fragment of ColE1 containing the ColE1 inverted repeat between the EcoRI and BamHI sites of pBR322 (26). pColIR315 is a deletant of pColIR215, created by taking the ColE1 insert and ligating it between the EcoRI and BamHI sites of pAT153. pIRbke8 was constructed by cloning two ligated synthetic oligonucleotides into the BamHI site of pAT153 (27). Maps of pColIR315 and pIRbke8 showing the locations of restriction targets relevant to the present work and the main inverted repeats (filled boxes), together with the base-sequences of these inverted repeats, are shown in figure 1.

pColIR215 was prepared from E. coli HB101 grown at A_{600} = 0.6 for 16 hr in the presence of 150 µg/ml chloramphenicol. Cells were lysed with lysozyme, EDTA and Triton X100, and the cleared lysate centrifuged with CsCl and ethidium bromide. Supercoiled plasmid DNA was recovered by side puncture,

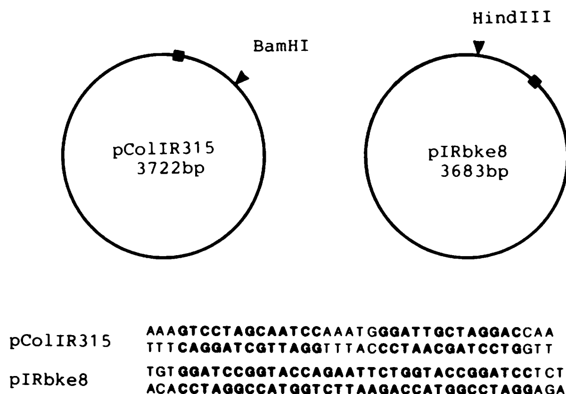


FIGURE 1

pColIR315 and pIRbke8, the plasmids used in these studies. The locations of the major inverted repeated (filled boxes) are shown with respect to the restriction targets used to test for site-specific nuclease cleavage at the cruciform. pColIR215 is identical to pColIR315 except for an insertion of 705 base pairs beginning at 1708 bp relative to the unique EcoRI site. The sequences of the inverted repeats are indicated below the plasmid maps - nucleotides related by two-fold rotational symmetry are shown in larger type.

extraction with butan-1-ol, extensive dialysis against 10 mM Tris, pH 7.5, 0.1 mM EDTA, precipitation with ethanol and redissolving in water. Cruciform free pColIR315.Q and pIRbke8.Q were prepared exactly as for pColIR215, but were recentrifuged in 10 mM Tris pH 7.5, 0.1 mM EDTA, 1 mg/ml CsCl, 250 μg/ml ethidium bromide at 15°C for 16 hr at 54,000 rpm in a Beckman VTi 65 rotor. DNA was recovered by side puncture and the solution immediately cooled on ice, before repeated extractions with ice-cold butan-1-ol. The DNA was then dialysed against pre-cooled 10 mM Tris pH 7.5, 0.1 mM EDTA at 6°C. These DNA stocks were then stored at -70°C, care being taken to thaw them below 15°C when required.

Enzymes

S1 nuclease (Bethesda Research Labs) was diluted to a 3550 U/ml stock solution, and incubations performed in 50 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$ at 15°C for 20m in 20 μl volumes containing 3U S1 nuclease. T4 endonuclease VII was a gift from Dr B. Kemper. Incubations were performed in 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 250 μg/ml bovine serum albumin at 20°C for 60m in 20 μl volumes containing 500U T4 endonuclease VII. Restriction enzymes (Bethesda Research Labs) were used as directed by the manufacturer.

Kinetic measurements

DNA samples were incubated at a given temperature in a Haake D8 thermostatted bath, to an accuracy of ± 0.05 C deg. At the end of the incubation time the sample, or an aliquot, was quickly transferred to ice. Uncertainties in cooling times probably limit temporal accuracy to ± 10s. Once ice cold, DNA samples were incubated with either S1 nuclease or T4 endonuclease VII before being precipitated with ethanol. The pelleted DNA was redissolved in water and digested to completion by a restriction enzyme. The digested plasmid was electrophoresed in 1% agarose in 90 mM Tris, pH 8.3, 90 mM borate, 10 mM EDTA at ambient temperature for 16h. Gels were stained in 1 μg/ml ethidium bromide, destained in water and photographed under ultraviolet light using Kodak Tri-X Pan film. Quantitation of results was achieved by densitometry and

integration of photographic negatives. All slopes were calculated using linear regression procedures.

RESULTS

Removal of cruciform structures from supercoiled pColIR215

In order to study the extrusion of cruciform structures in supercoiled plasmids it was necessary to be able to remove them quantitatively. The rationale behind the approach used in these studies is illustrated in figure 2. Supercoiled DNA, with cruciforms potentially extruded, are positively supercoiled by intercalation of ethidium bromide. Since the existence of cruciform structures requires negative supercoiling (3-21) they will be destabilised and hence removed from the DNA. The ethidium bromide is then partitioned into the organic phase by repeated extractions with butan-1-ol on ice. Removal of the intercalator results in a reversion to negative supercoiling. However, as I demonstrate below, this species has not re-extruded a cruciform, and will be referred to as pColIR315.Q.

An example of cruciform removal from supercoiled pColIR215 is shown in figure 3. Plasmid was exposed to a range of ethidium bromide concentrations followed by cold butan-1-ol

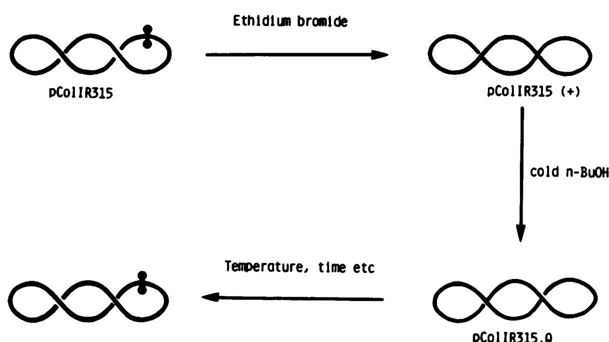


FIGURE 2

Scheme to show the protocol used to isolate pColIR315.Q ie plasmid devoid of cruciform structure. Supercoiled plasmid was exposed to ethidium bromide to generate positively supercoiled DNA, pColIR315 (+). Upon removal of the intercalator by solvent extraction, the DNA reverts to negatively supercoiled species free of cruciforms, pColIR315.Q.

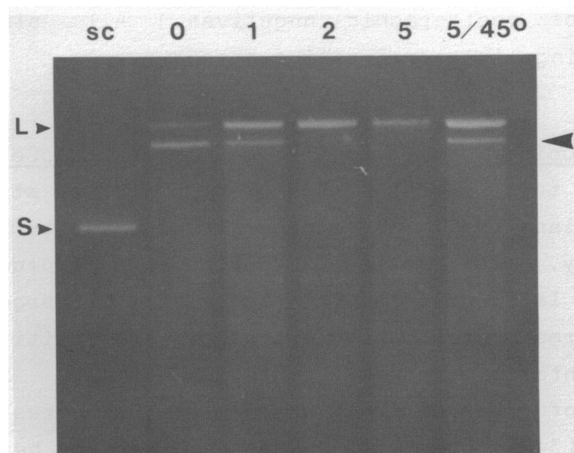


FIGURE 3

Removal of the ColE1 cruciform from pColIR215 as a function of ethidium bromide concentration. Supercoiled pColIR215 was incubated with various concentrations of ethidium bromide in 10 mM Tris, pH 7.5 for 60m at 25°C before cooling on ice and extracting five times with cold butan-1-ol. One sample (incubated with 5 µg/ml ethidium bromide) was heated at 45°C for 5m and returned to ice. DNA samples were assayed for cruciform formation by S1 nuclease digestion at 15°C followed by complete BamHI digestion and 1% agarose gel electrophoresis. Site-specific S1 nuclease cleavage at the ColE1 cruciform generates a fragment of 4087 bp which migrates as the well resolved band indicated by the arrow drawn on the right. Full length linear (form III DNA) pColIR215 is indicated by the arrow labelled L, while supercoiled plasmid (form I DNA) is indicated by S. Gel tracks contained: SC, supercoiled pColIR315; 0 to 5, pColIR215 incubated with that concentration (µg/ml) of ethidium bromide before S1 nuclease and BamHI cleavages; 5-45° pColIR215 treated with 5 µg/ml ethidium bromide and exposed to 45°C for 5m before assaying as the other samples.

extraction. The presence of the ColE1 cruciform was tested for by cleavage with S1 nuclease at 15°C followed by incubation with BamHI. Site-specific S1 nuclease cleavage at the loop of the ColE1 cruciform would generate a 4087 bp fragment which is clearly resolved from the 4427 bp full length linear species. It can be seen from figure 3 that the ColE1 cruciform is extruded in plasmids subjected to ethidium bromide concentrations of 1 µg/ml or below. Above this concentration, however, the 4087 bp fragment is absent, indicating that the

cruciform structure is not present in the supercoiled DNA. This is not a permanent removal due, for example, to trace quantities of ethidium bromide remaining, since the S1 nuclease sensitivity at the ColE1 cruciform may be regained by simply heating this DNA to 45°C for five minutes, as shown in figure 3. This recovery of the cruciform structure on warming forms the principal subject of investigation in the remainder of this paper.

For preparing larger quantities of cruciform free plasmids, DNA is normally taken directly from a CsCl-ethidium bromide gradient, extracted repeatedly with butan-1-ol and finally dialysed against 10 mM Tris pH 7.5, 0.1 mM EDTA. Stock solutions of approximately 500 µg/ml are easily obtained of DNA which has not been subjected to potentially perturbing conditions such as phenol extraction, ethanol precipitation or warming above 7°C.

The ColE1 cruciform can re-extrude when the temperature is raised

It is clear from the experiment described above that an exposure to 45°C for 5m is sufficient to allow the ColE1 cruciform of pColIR215.Q to extrude. Figure 4a shows a finer analysis of this temperature dependence for pColIR315.Q. Aliquots of native supercoiled pColIR315.Q in 10 mM Tris pH 7.5 were separately exposed to temperatures between 30 and 37°C (this range was selected after initial coarser experiments) in one degree intervals for five minutes, and then placed back on ice. It is important to note that from this point onward these samples were treated identically. Each sample was incubated with S1 nuclease at 15°C followed by complete BamHI digestion. It is evident that very little cruciform extrusion has occurred in the samples exposed to 32°C or lower (judged by the absence of a 3382 bp band below the 3722 bp full length linear species), whilst higher temperatures have allowed extrusion to occur. For a 5m exposure, 33°C seems to be a critical temperature.

These results are not dependent upon the choice of probe. T4 endonuclease VII is a highly structure-specific probe for the four-way junction, cleaving at the base of the cruciform

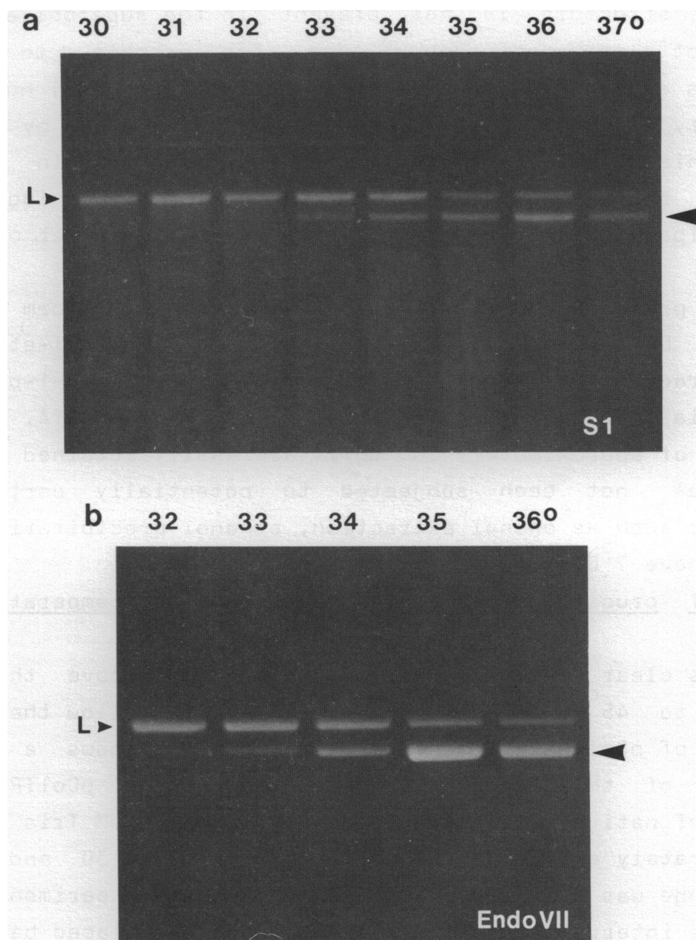


FIGURE 4

Extrusion of the pColIR315 cruciform as a function of temperature. Aliquots of pColIR315.Q were incubated with 10 mm Tris pH 7.5 at the indicated temperatures (°C) for 5 min before returning to ice. Cruciform structure formation was assayed either by S1 nuclease at 15°C (a) or T4 endonuclease VII at 20°C (b), each followed by complete BamHI digestion and 1% agarose gel electrophoresis. Site-specific cleavage at the ColE1 cruciform generates a 3382 bp DNA fragment migrating as the band indicated by the arrow drawn on the right. Full length linear pColIR315 is indicated by the arrow labelled L.

stems (12). Analogous experiments to those above were performed on pColIR315.Q, except that the S1 nuclease step was replaced by an incubation with T4 endonuclease VII at 20°C.

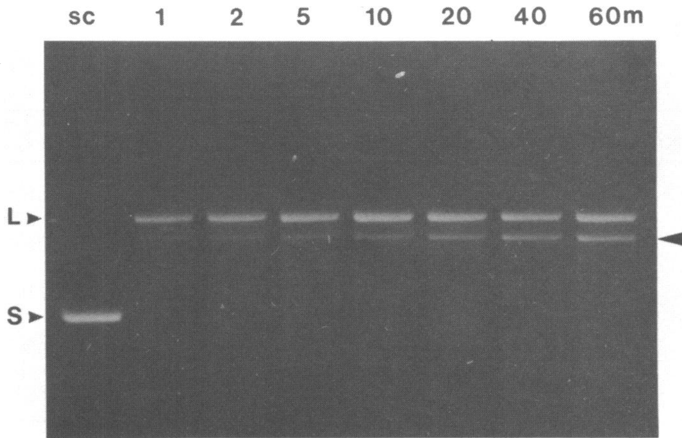


FIGURE 5

Extrusion of the pColIR315 cruciform at 33°C as a function of time. pColIR315.Q was incubated with 10 mM Tris pH 7.5 at 33°C and samples withdrawn and immediately cooled on ice at the times indicated (minutes). Cruciform structure formation was assayed by S1 nuclease digestion at 15°C and BamHI cleavage followed by 1% agarose gel electrophoresis.

The results obtained, shown in figure 4b, are essentially identical to those using S1 nuclease, once again revealing the critical extrusion temperature of 33°C. Clearly these two very different probes revealing a structural change brought about during the heating step ie the extrusion of the ColE1 cruciform.

In the light of studies of different inverted repeats (13,20,24,25) it seemed probable that the observed temperature dependence of cruciform extrusion by the ColE1 sequence reflected a kinetic barrier. That this is indeed the case may be shown by studying the time dependent formation of the cruciform structure at various temperatures. As an example, figure 5 shows the extrusion of this cruciform at 33°C, monitored by the formation of the S1 nuclease hypersensitive site. At this temperature the cruciform is extruded gradually over the course of an hour, and the disappearance of non-cruciform DNA exhibits logarithmic dependence of concentration on time, corresponding to a first order process characterised by a rate constant $k = 2.17 \times 10^{-4}\ s^{-1}$.

Table 1 Rate constants for ColE1 cruciform extrusion in pColIR315 in 10 mM Tris pH 7.5 between 28 and 36°C. The numbers in parenthesis are standard errors for the kinetic data. Half times are calculated from t1/2 = 0.69/k.

Temperature (°C)	k (s^{-1})	t1/2
28	2.56 (0.14) x 10^{-6}	74h
30	6.41 (0.25) x 10^{-6}	29h
32	4.08 (0.08) x 10^{-5}	47m
34	6.63 (0.70) x 10^{-4}	17m
36	4.45 (0.18) x 10^{-3}	2.6m

These data held a number of surprises. Firstly the temperature required to obtain relatively rapid extrusion was much lower that had been reported for different inverted repeat sequences (13,20). In particular, Gellert et al (20) had observed slow extrusion (ie half lives measured in hours) by two sequences even at 50°C. Thus it seemed probable that different DNA sequences might possess different kinetic properties. Secondly, the temperature coefficient for the ColE1 extrusion is extremely large. Rate constants and half times as a function of temperature between 28 and 36°C are presented in table 1, and it can be seen that the rate constants vary by about six natural logarithms in 8 C deg. These data correspond to a huge enthalpy of activation ($\Delta H^{\ddagger}$) and hence a very large entropy of activation ($\Delta S^{\ddagger}$) must be present also since the reaction proceeds rapidly above 35°C. The large $\Delta H^{\ddagger}$ points to a highly cooperative mechanism for the extrusion reaction, which I discuss further below.

<u>Extrusion of the ColE1 cruciform is suppressed at elevated ionic strength</u>

Kinetic studies of cruciform extrusion by different inverted repeats have suggested that ionic strength is an important determinant of reaction rate (20,24). The effect of various concentrations of sodium chloride on the extrusion of the ColE1 inverted repeat of pColIR315.Q was therefore examined. A series of pColIR315.Q aliquots were incubated in

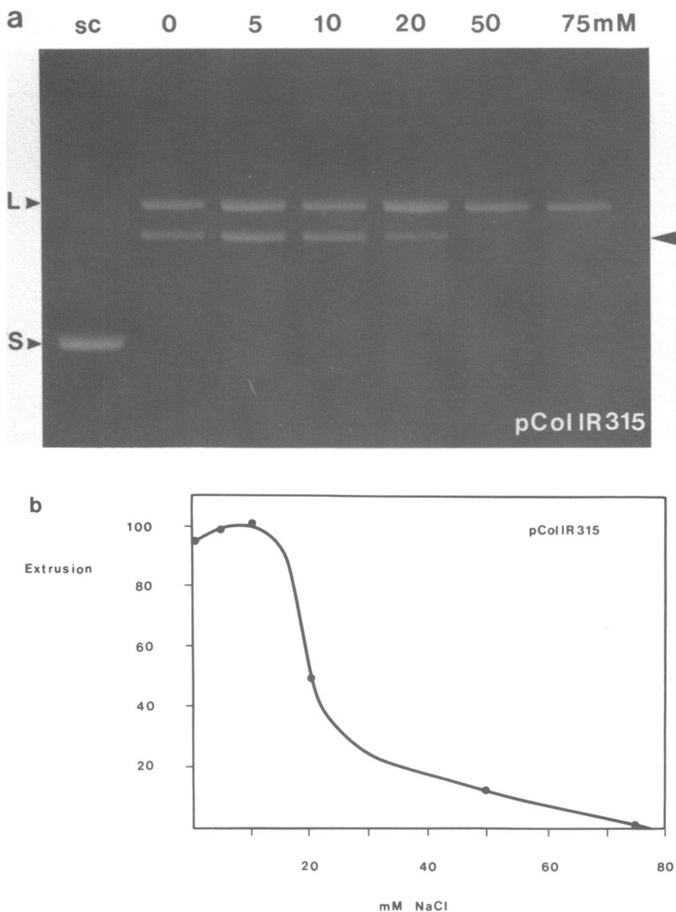
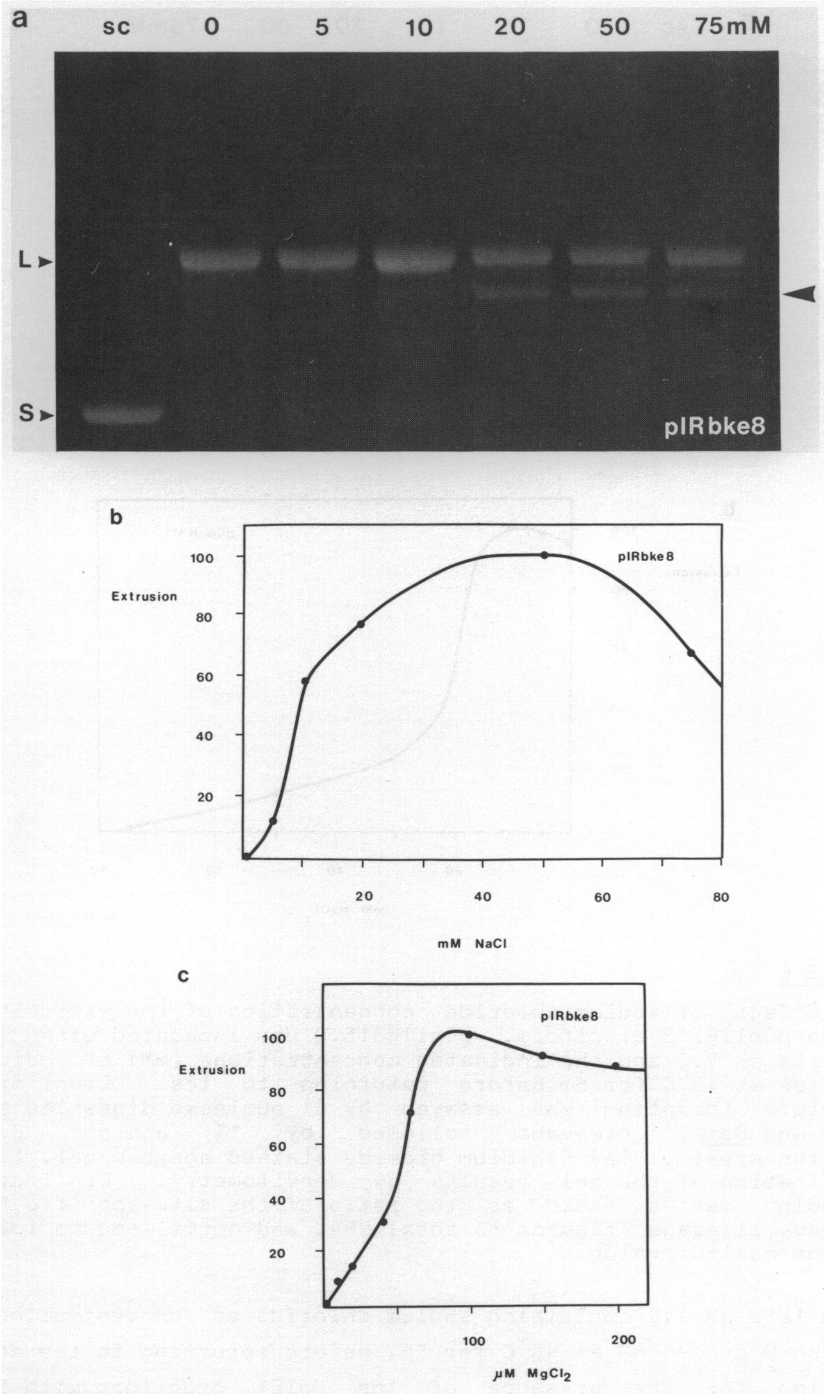


FIGURE 6

Effect of sodium chloride concentration of the extrusion of the pColIR315 cruciform. pColIR315.Q was incubated with 10 mM Tris pH 7.5 and the indicated concentrations (mM) of sodium chloride at 45°C for 5m before returning to ice. Cruciform structure formation was assayed by S1 nuclease digestion at 15°C and BamHI cleavage followed by 1% agarose gel electrophoresis. (a) Ethidium bromide stained agarose gel. (b) Quantitation of the gel results by densitometry. Cruciform extrusion was assessed as the ratio of the site-specific S1 nuclease cleavage fragment to total DNA, and normalised to 100% for the maximum value.

10 mM Tris pH 7.5 containing sodium chloride at concentrations between 0 and 75 mM at 45°C for 5m, before returning to ice and assaying for the presence of the ColE1 cruciform with S1


a
sc
0
5
10
20
50
75mM
L
S
pIRbke8
b
100
Extrusion
80
60
40
20
pIRbke8
20
40
60
80
mM NaCl
c
pIRbke8
100
Extrusion
80
60
40
20
100
200
μM MgCl2

nuclease as before. The results of such an experiment are shown in figure 6. It can be seen that in contrast to the results reported for other sequences (20,24), the extrusion of the ColE1 cruciform is actually suppressed as the concentration of sodium chloride is raised. Indeed, 75 mM sodium chloride appears to abolish completely the extrusion reaction at 45°C. Magnesium chloride behaves qualitatively very similarly, except that the abolition of the extrusion occurs by about 25 mM. Near maximal extrusion rates are obtained in the absence of ions, either monovalent or divalent.

The extrusion of the synthetic inverted repeat of pIRbke8.Q requires the presence of cations

pIRbke8.Q was prepared by identical procedures to those for pColIR315.Q. It was first observed that when this DNA was incubated in 10 mM Tris pH 7.5, identical conditions to those used for pColIR315.Q, no cruciform extrusion could be detected even up to 65°C. It was then discovered that for this sequence, inclusion of salt was an essential requirement for the extrusion reaction. This is illustrated by the experiment shown in figure 7a, in which pIRbke8.Q was exposed to 45°C for five minutes in the presence of various sodium chloride concentrations, before returning to ice and performing an S1 nuclease assay exactly as before. The gel shows that no extrusion occurred in the absence of salt, but that there was a smooth increase in extrusion up to the maximal extent at 50 mM sodium chloride. This behaviour is in marked contrast to that of pColIR315.Q, the results being almost the exact inverse of

FIGURE 7

Effect of sodium chloride and magnesium chloride on the extrusion of the pIRbke8 cruciform. pIRbke8.Q was incubated with 10 mM Tris pH 7.5 and the indicated concentrations of salt at 45°C for 5m before returning to ice. Cruciform structure formation was assayed by S1 nuclease digestion at 15°C and HindIII cleavage followed by 1% agarose gel electrophoresis. (a) Ethidium bromide stained agarose gel of pIRbke8.Q extrusion as a function of sodium chloride concentration (mM). (b) Quantitation of results from (a) by densitometry. (c) Extrusion of pIRbke8.Q as a function of magnesium chloride concentration (μM). In both (b) and (c) extrusion was assessed at the ratio of the site-specific S1 nuclease cleavage fragment to total DNA, normalised to 100% for the maximum value.

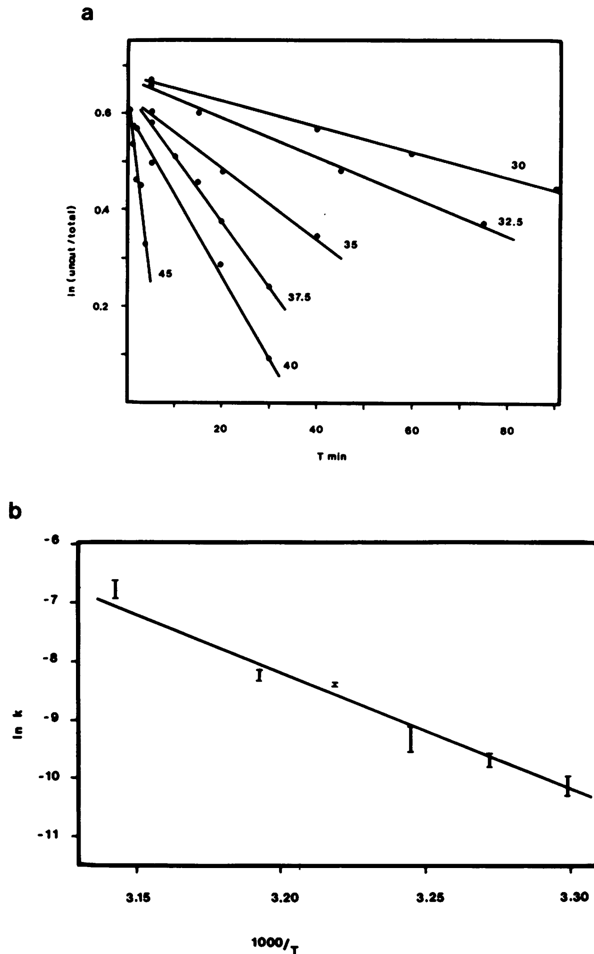


FIGURE 8

8. The kinetics of pIRbke8 cruciform extrusion as a function of temperature. (a) pIRbke8.Q samples were incubated in 10 mM Tris pH 7.5 at a given temperature between 30 and 45°C and aliquots withdrawn to ice at various time intervals. These were assayed for the presence of cruciform structure by S1 nuclease at 15°C and HindIII cleavage. The fraction of unextruded molecules was measured by gel electrophoresis and densitometry, and the natural logarithm plotted as a function of time of incubation. Each set of data points was subjected to linear regression. They are labelled with their extrusion reaction temperatures (°C). (b) Arrhenius plot of the rate constants measured in (a) against the reciprocal absolute temperatures. The error bars indicate the standard errors on the kinetic data.

Table 2 Rate constants for pIRbke8 extrusion in 10 mM Tris pH 7.5, 50 mM NaCl between 30 and 45°C.

Temperature (°C)	k (s^{-1})	t1/2
30	4.00 (0.63) x 10^{-5}	4.8h
32.5	6.18 (0.71) x 10^{-5}	3.1h
35	9.06 (1.90) x 10^{-5}	2.1h
37.5	2.25 (0.07) x 10^{-4}	51m
40	2.63 (0.27) x 10^{-4}	44m
45	1.16 (0.18) x 10^{-3}	9.9m

each other. Magnesium chloride is very much more effective in promoting cruciform extrusion of pIRbke8.Q, shown in figure 7c. ∿100 μM magnesium chloride is sufficient to achieve maximal extrusion of pIRbke8.Q at 45°C.

Activation parameters for pIRbke8

The extrusion kinetics for pIRbke8.Q at the optimal salt concentration of 50 mM sodium chloride have been measured as a function of temperature. Figure 8a shows that between 30 and 45°C there is a steady increase in the rate of extrusion, and rate constants have been calculated for each temperature and are given in Table 2. These are presented in the form of an Arrhenius plot in figure 8b. Within the experimental error (the error bars are obtained from the standard errors of the rate constants ie the gradients of the lines in figure 8a), these data are consistent with a single exponential

$$k_r = e^{-Ea/RT} \quad (1)$$

where k_r is the rate constant, R is the gas constant and T the absolute temperature. The Arrhenius activation energy is calculated to be

$$Ea = 42.4 \pm 3.2 \text{ kcal mole}^{-1}$$

Whilst this is a high activation energy, it is less than a quarter of the value which is derived from the pColIR315.Q data in 10 mM Tris, although the very high temperature coefficient

for the latter cruciform makes the actual value difficult to measure with accuracy.

Since the extrusion of a cruciform is a unimolecular reaction, the enthalpy of activation is given by

$$\Delta H^{\ddagger} = Ea - RT \qquad (2)$$
$$= 41.8 \text{ kcal mole-1}$$

The entropy of activation ($\Delta S^{\ddagger}$) at a given temperature is calculated by application of standard transition state theory (28),ie

$$k_r = \frac{kT}{h} e^{\Delta S^{\ddagger}/R} e^{\Delta H^{\ddagger}/RT} \qquad (3)$$

where the terms k and h in the pre-exponential expression are the Boltzmann and Planck constants respectively. At 37°C

$$\Delta S^{\ddagger} = 59 \text{ cal deg-1}$$

for the pIRbke8 cruciform.

DISCUSSION

The basic conclusions which have emerged from these results may be stated simply.

1. Two inverted repeat sequence exhibit substantial kinetic barriers in their extrusion reactions.
2. Detailed kinetic properties are a function of DNA base sequence.
3. The kinetic properties are unaffected by the probing methods employed.

Major kinetic barriers have been identified for the extrusion of a number of inverted repeats (13,20,24,25). In some cases (13,20) extrusion rates have been found to be very slow (half times in hours) even at 50°C. In other instances (24,25) relatively fast extrusion rates have been observed at 37°C. The present study gives two more examples of inverted repeats which, despite large activation barriers, may nevertheless extrude rapidly at 37°C given optimal salt conditions. Is there a discrepency between these two groups of studies? We can probably exclude differences in experimental technique ie in the cruciform removal procedures. Essentially similar protocols have been employed by all groups of workers

ie incubation with ethidium bromide followed by cold solvent extraction. We have been scrupulously careful to avoid any helix destabilising conditions including phenol extraction, ethanol precipitation or warming. A second possibility is that differences in plasmid topology may be significant. The bulk of the studies of Courey and Wang (13) were performed on plasmid DNA of specific linking difference (σ) of -0.03, and when experiments were carried out on pAC103 using a mixture of topoisomers between σ = -0.045 and -0.075, reaction times of approximately one hour at $37^{\circ}C$ were obtained. If the extrusion mechanism induces a negative twist change (ΔTw) in the transition state, then we would predict that the extrusion should proceed faster with reduction in linkage. This has in fact been observed for the ColE1 cruciform in pAO3 (25). However, this cannot be a complete explanation for these differences, since Gellert and co-workers (20) observe slow kinetics at $50^{\circ}C$ for the ΔLk = -13 topoisomer of pUC7, corresponding to σ = -0.047.

We are therefore required to consider the possibility that the kinetic properties of cruciforms are determined by the DNA base sequences of the inverted repeats. This is bourne out by the studies presented here. Two very similar plasmids, pColIR315 and pIRbke8, have been prepared using identical protocols, and yet these cruciforms possess reproducibly different kinetic properties. These differences must be due to differences in DNA sequence, either those of the inverted repeats themselves, or the flanking regions. This distinction between these two sequences is particularly acute with regard to salt dependence. The behaviour of the synthetic inverted repeat of pIRbke8 appears to be closely similar to that reported for pUC7 (20) and pOCE12 (24), all three having optimal extrusion kinetics at or close to 50 mM sodium chloride. The very different salt dependence of the ColE1 sequence is presently without precedent, and could be a consequence of a different reaction mechanism. That it is the ColE1 sequence which is exceptional is also indicated by the exceedingly high temperature dependence of the extrusion of this cruciform. The Arrhenius activation energy measured for

pIRbke8 (42.4 kcal mole^{-1}) is of similar magnitude to those reported for pOCE12 (33 kcal mole^{-1}) (24) and pUC7 (approximately 50 kcal mole^{-1}) (20), whereas that of ColE1 is several times larger than this value in the absence of sodium chloride. Preliminary experiments with plasmids containing inverted repeats of sequence different from either ColE1 or pIRbke8 indicate that their properties are more like the latter in general. Just what sequence features give rise to the atypical kinetic properties of the ColE1 sequence are not yet clear, but the general point remains viz that the kinetic characteristics of different cruciforms are a function of base sequence. We have recently studied an $(AT)_{34}$ sequence from a Xenopus globin gene which forms a cruciform with rapid kinetics even at very low temperature when supercoiled (D. Greaves, R. Patient and D.M.J. Lilley, in preparation). This further illustrates the sequence-dependent nature of cruciform extrusion kinetics.

Could the choice of the method used to assay for the presence of the cruciform influence the results obtained? Different approaches employed include topological band shift methods (9,13,19-21), psoralen cross-linking (15) and inhibition of restriction endonucleolysis (7,13,29), in addition to the S1 nuclease and T4 endonuclease VII probes used in this study. It has been suggested (13,20) that S1 nuclease might itself influence extrusion kinetics by facilitating the formation of a cruciform. We have demonstrated previously (21) that nuclease and chemical probes do not affect the thermodynamic stability of the ColE1 cruciform, and have now shown that the kinetic properties are similarly unaffected. The data of figure 4, for instance, show unequivocally that it is the heating step prior to the S1 nuclease or T4 endonuclease VII assay step which causes the cruciform extrusion, ie the generation of the hypersensitive site. If the heating step is insufficient to cause extrusion, then the nuclease assay does not detect site-specific structural perturbation. It should also be noted that identical results are obtained using S1 nuclease and T4 endonuclease VII, despite their contrasting modes of cleavage and reaction conditions. A major advantage

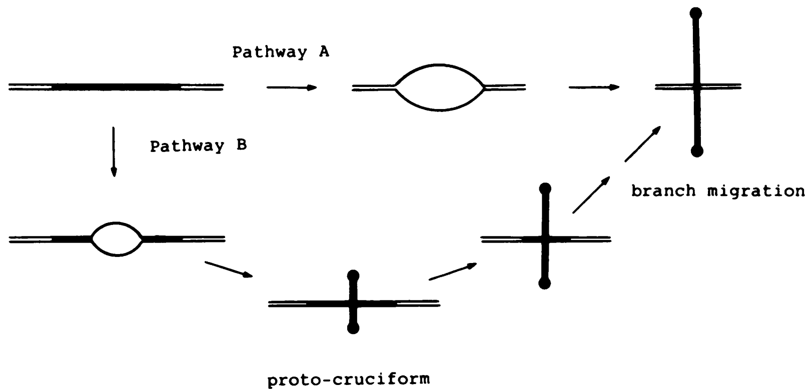


FIGURE 9

Schematic illustration of two possible mechanistic pathways for cruciform extrusion. The filled portion represents the full extent of the inverted repeat. In pathway A this is completely melted to form a large bubble which undergoes intrastrand base-pairing to the complete cruciform structure. Pathway B is initiated by formation of a smaller centralised bubble, followed by formation of the primary 'proto-cruciform' and branch migration to the complete cruciform. Note that in contrast to pathway A, in B the extreme nucleotides of the inverted repeat do not participate in structural reorganisation until the final stages.

of nuclease probes is the positional information obtained ie the site at which cruciform extrusion occurs, in contrast to the global information forthcoming from band shift methods. Obviously if nuclease digestion experiments are performed at higher temperatures, such that significant cruciform extrusion may occur during the course of the incubation, then the results obtained no longer simply reflect the population of conformational species existing at the outset. This may very well be true for nuclease digestions carried out at 37°C, which was the case for many earlier experiments. We now routinely perform S1 nuclease and T4 endonuclease experiments at 15 or 20°C, and both enzymes are active even on ice.

Can we identify a likely mechanism for cruciform extrusion? Previously (27) we have formulated two potential alternative pathways, which are drawn schematically in figure 9. Pathway A requires helix opening along the entire length of the inverted repeat, followed by a concerted process of

intra-strand duplex formation ('snapback'). Pathway B contains many more steps, involving the initial formation of a smaller central unwound 'bubble', formation of a proto-cruciform, and sequential formation of inter- and intra-strand bonding via a random walk branch migration scheme. Of course we may only speculate at present as to how realistic either mechanism may be, and the real situation may very well be more complex. However, these mechanisms do offer at least a partial explanation of the available data. First we will consider the likely kinetic consequences of mechanism A. This involves passing through a state in which three or more helical turns are completely disrupted - certainly a high energy state and probably closely similar to the reaction transition state. Since so many hydrogen bonds and stacking interactions are broken, this will result in a large enthalpy of activation ($\Delta H^{\ddagger}$), and the resulting disorder will necessitatea large entropy of activation ($\Delta S^{\ddagger}$). This will give rise to kinetic properties typical of a cooperative process. Since the melting temperature (Tm) of DNA is reduced at low ionic strength (30), the free energy of this transition state should be reduced in the absence of salt, resulting in a faster reaction rate. Mechanism B is more difficult to assess, as the transition state is harder to identify. It should precede the branch migration steps, since these involve transfer of hydrogen bonds between isoenergetic states (ignoring the torsional relaxation imparted as the cruciform arms elongate), and experimentally branch migration has been observed to be a relatively fast process (31). We will assume, therefore, that the transition state lies between the initial bubble and the proto-cruciform. Less base pairs need be disrupted, and hence both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are reduced. However, if the four-way junction is beginning to assemble in the transition state, then phosphate-phosphate repulsion may become significant, and hence the free energy of this state may be reduced by increased salt concentration. Beyond the optimal concentration required to achieve this however, further increases will make the initial bubble formation more difficult, thus giving rise to a maximum in the salt dependence. Divalent cations are particularly

effective in this kind of structural role, as seen in the crystal structure of tRNA for example (32-34). It can be seen that the kinetic properties predicted from these two mechanisms actually fit the experimental observations quite well ie ColE1 extruding via pathway A and pIRbke8 (and probably the majority of sequences) extruding via pathway B. This is presently wholly speculative, however, and requires a great deal more investigation. Neither mechanism invokes any role for the flanking DNA sequences. One striking feature of the ColE1 sequence is that whilst the inverted repeat itself is overall 52% A + T, the 200 bp in which it is embedded is 77% A + T, an abnormally high A + T content. It is possible that longer range structural changes may participate in the extrusion process, and that these may even influence the kinetic pathway adopted.

Can any biological conclusions be drawn from these kinetic experiments? Cruciform extrusion kinetics are a complex function of temperature, ionic conditions, superhelix density and, significantly, DNA base-sequence. It would therefore be premature to pronounce on the in vivo potential for cruciform formation. The current climate of opinion is against a cellular role for cruciform structures for two main reasons. First, experiments designed to detect cruciforms in vivo have failed to support their existence (13,15,20,35), and second, kinetic results have been extrapolated to suggest that all cruciforms will be kinetically forbidden in vivo. The results obtained here indicate that it may be misleading to generalise kinetic results obtained from a few examples since these properties may be profoundly sequence-dependent. It may well be that cruciform structures are kinetically barred in vivo. For example, if the effective superhelix density of the bacterial chromosome is lowered (by protein binding, for instance), then it is quite possible that extrusion rates at $37^{o}C$ may be much longer than the cell doubling time for a given sequence. However, it is not justified to conclude that all sequences are necessarily kinetically forbidden, based upon data presently available. We are presently embarking on a systematic analysis of cruciform extrusion kinetic properties

as a function of DNA base-sequence in order to attempt to deduce the general principles involved, and hopefully to test the potential reaction mechanisms proposed.

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