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The free energy of DNA supercoiling is enthalpy-determined

(ColE1 *amp* plasmid/thermodynamics of supercoiling/microcalorimetry/torsional and flexural rigidity/topoisomerase I)

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ABSTRACT **The thermodynamics of superhelix formation was determined by combining superhelix density data with enthalpy values obtained from microcalorimetric measurements of the relaxation of supercoiled ColE1 *amp* plasmid DNA in the presence of topoisomerase I from *Escherichia coli* (ω protein). The thermodynamic quantities for superhelix formation at 37°C in 10 mM Tris/2 mM $MgCl_2$/1 mM EDTA pH 8, are: ΔG = 921 kJ·(mol of plasmid)$^{-1}$; ΔH 2260 kJ·(mol of plasmid)$^{-1}$; ΔS = 4.3 kJ·(mol of plasmid·K)$^{-1}$. These data clearly demonstrate that the unfavorable Gibbs free energy associated with supercoiling of DNA results exclusively from the positive enthalpy involved in formation of superhelical turns. A positive overall entropy change accompanies superhelix formation, which overcompensates the expected decrease of configurational entropy. By neglecting contributions from bending, an estimate of the torsional rigidity $C = 1.79 \times 10^{-19}$ erg·cm (1 erg = 0.1 μJ) of the supercoiled ColE1 *amp* plasmid DNA was made on the basis of the enthalpy value. This value is in excellent agreement with values of C derived from subnanosecond time-resolved fluorescence depolarization measurements for pBR322 DNA [Millar, D. P., Robbins, R. J. & Zewai, A. H. (1982) *J. Chem. Phys.* 76, 2080–2094]. The magnitude of C is larger than for linear DNAs, indicating that supercoiled DNA is more rigid than linear DNA.**

As a result of topological constraints, circular duplex DNA exhibits unique structural and energetic properties that lead to enhanced reactivity in a large number of biological processes (1). It has been well established that many functions of DNA in replication (2, 3), transcription (4–12), recombination (13), and repair (14, 15) depend critically on the degree of its superhelicity. Supercoiling favors binding of single-strand reagents and the occurrence of cruciform structures in palindromic DNA sequences (16–19). Several of these exceptional features of superhelical DNA can be understood if one recalls that any reaction resulting in a reduction of superhelix density can use the decrease in Gibbs free energy associated with that process. These aspects were realized very early (20–27) and have initiated both experimental and theoretical work to determine the Gibbs free energy of supercoiling by various methods involving interaction of dyes (28, 29), alkaline buoyant titration (30), and band counting in gel electrophoresis (31–34). Although all of these methods permitted accurate determination of the superhelix density and, thus, calculation of the Gibbs free energy, no proper estimates of the enthalpic contribution to the Gibbs free energy of supercoiling could be made on the basis of those studies. Knowledge of the enthalpy of superhelix formation is desirable for obtaining insight into the elastic properties of DNA, for checking predictions of theoretical studies, and for precise partitioning of the Gibbs free energy into enthalpic and entropic contributions.

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MATERIALS AND METHODS

Preparation of ColE1 *amp* RSF2124 Plasmid DNA. The plasmid was isolated from the bacterial strain *Escherichia coli* M609 (a gift of R. Mattes, Boehringer, Tutzing) by the method of Burkardt *et al.* (35) except for the cell lysis, where the procedure of Voordouw *et al.* was followed (36). Covalently closed circular DNA was separated from linear and open circular DNA in the presence of ethidium bromide (0.5 μg/ml) by using a cesium chloride gradient (Hitachi vertical rotor RPV 65T; 48 hr at 42,000 rpm and 15°C).

Preparation of the Topoisomerase I (ω protein). ω protein was isolated from frozen *E. coli* cells by the procedure by Wang (37) with modifications introduced by H. P. Vosberg (Max Planck Institut, Heidelberg; personal communication). The modifications comprised purification steps using phosphocellulose chromatography, hydroxylapatite chromatography, and DNA cellulose chromatography. The DNA cellulose was prepared as described by Litman (38). Protein concentration was determined by the method of Bradford (39).

ω Protein Assay. The activity of the topoisomerase I was controlled by using a standard assay mixture of 0.01 M Tris (pH 8.0)/0.002 M $MgCl_2$/0.001 M EDTA containing 15 μg of DNA in 1 ml; 0.1- to 5-μl samples of the fraction containing ω protein were added to 20 μl of the assay mixture, and the solution was incubated at 37°C for 30 min. The reaction was stopped by adding 5 μl of a 1% *N*-lauroylsarcosine solution. The resulting solutions were analyzed by horizontal ethidium bromide/agarose gel electrophoresis to detect the amount of ω protein necessary to relax fully the 0.3 μg of DNA in the assay mixture.

Agarose Gel Electrophoresis. Electrophoresis was used for qualitative and quantitative characterization of the plasmid DNA configuration; 19 × 19 × 0.5 cm horizontal slab gels were used, containing 0.7% agarose and 0.4 μg ethidium bromide per ml. The electrophoresis buffer consisted of 0.089 M Tris, 0.089 M boric acid, 0.0025 M EDTA, and 0.4 μg of ethidium bromide per ml (pH 8.0). A constant current of 45 mA was applied for 14 hr.

Determination of the Percentage of Closed Circular DNA. The total DNA concentration was determined by UV absorption measurements at 260 nm of the sample solutions using an extinction coefficient at 260 nm of 200 $cm^2 \cdot mg^{-1}$. The percentage of closed circular DNA was obtained from scans at 250 nm of the negatives of polaroid photos taken of the agarose gels under UV illumination. The sample solutions used for calorimetry contained between 75% and 95% closed circular DNA.

Determination of Superhelix Density. Superhelix density was determined by cesium chloride density gradient centrifugation (40) and by fluorescence titration (41).

Calorimetry. An LKB batch microcalorimeter was used for the experiments. Measurements were performed at 37°C. With our conditions of thermostatting the instrument, heat pulses of 50 μJ can be well resolved. The sample cells were charged with approximately 4 ml of the plasmid DNA solu-

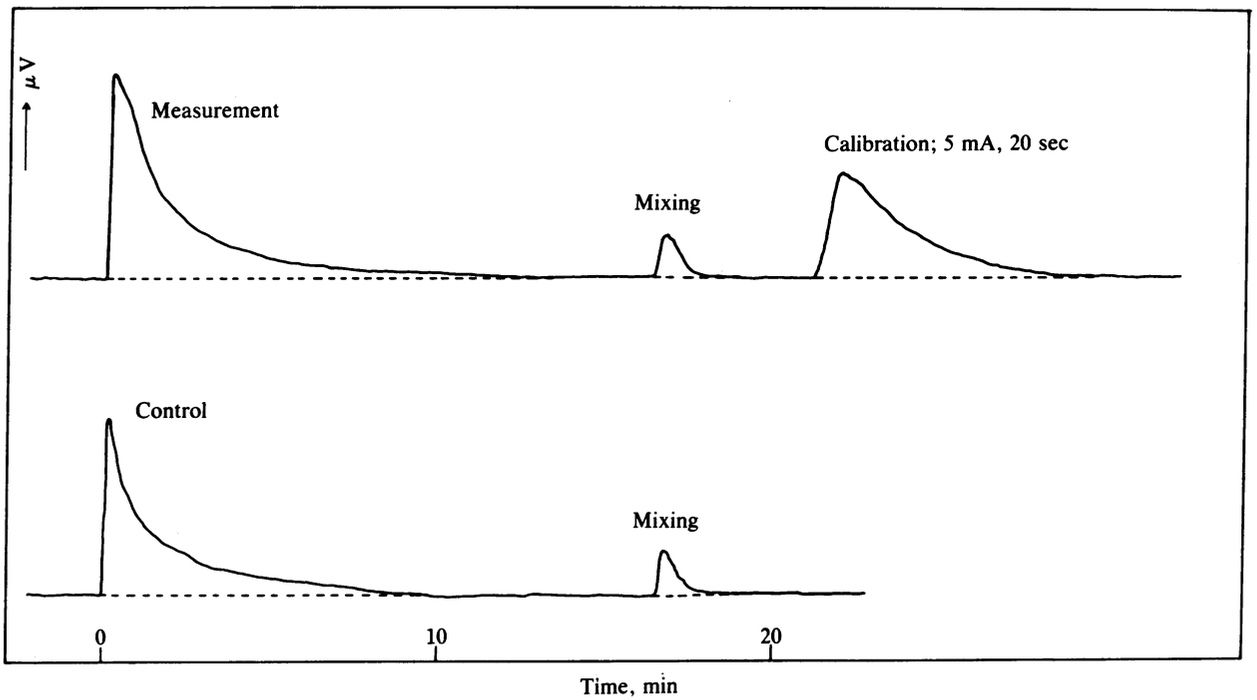

FIG. 1. Typical batch calorimetric measurements for relaxation of ColE1 *amp* plasmid DNA by using ω protein as catalyst. The figure shows tracings of measurement 1 in Table 1. The measurements were routinely followed by a series of mixing and calibration peaks; one of each is shown (*Upper*). The reference measurements have been performed with all four compartments filled with buffer. Separate dilution experiments of the DNA or the protein gave essentially the same results. The average of two or three such controls has been used for correction of the relaxation experiments.

tion. The reaction was started by mixing the DNA solution with 2 ml of the ω protein solution. Plasmid DNA and the ω protein were dialyzed to equilibrium against the reaction buffer (0.01 M Tris/0.002 M $MgCl_2$/0.001 M EDTA, pH 8.0). The DNA concentrations were approximately 0.5 mg/ml; the exact concentrations were determined as described above. For calculation of molar ΔH values, a molecular weight of 7.2×10^6 was used for ColE1 *amp* DNA. Heats of dilution of protein and DNA were negligible compared to the differential signal at 37°C of the buffer-filled sample and reference cells of the calorimeter. These differential signals were used for correction of the observed heats of reaction. As a control experiment, ω protein and open circular DNA solutions were mixed with no detectable heat effect. The protein concentrations used varied between 1 and 10 μg/ml. Thus, the highest molar protein/DNA ratio used was 1:1. However, usually DNA was in excess. Typical batch calorimetric measurements are shown in Fig. 1.

RESULTS

Enthalpy of Supercoiling. The enthalpy values obtained for relaxation of ColE1 *amp* DNA in the presence of topoisomerase I from *E. coli* are summarized in Table 1. The ΔH values have been corrected for the percentage of open circular and linear DNA present and refer to the reaction

ColE1 *amp*(closed circular, superhelical)

37°C ↓ ω protein

ColE1 *amp*(closed circular, relaxed).

The average value of six determinations is $\Delta H = -2260 \pm 409$ kJ/mol of plasmid. Analysis of the reaction mixtures by gel electrophoresis after the calorimetric measurements showed that besides the closed relaxed form of the circular DNA, also circular DNA molecules with nicks occurred. It is not clear whether these single-strand breaks are a result of the relaxation reaction or whether they are introduced only later during the prolonged standing (2 hr) of the reacted solution at 37°C in the calorimeter. This time can hardly be reduced due to mixing controls and calibration peaks. However, introduction of one or a few nicks per plasmid should have a negligible effect on the magnitude of the ΔH for superhelix relaxation in view of the small enthalpies of hydro-

Table 1. ΔH associated with relaxation of covalently closed circular (ccc) DNA

Exp.	DNA, mg	ccc DNA, %	ΔQ measurement, μJ	ΔQ correction, μJ	ΔH, kJ·(mol of plasmid)$^{-1}$
1	1.076	95	−1490	−1176	−2212
2	2.263	85	−1732	−1213	−1943
3	2.119	83	−2025	−1561	−1900
4	3.173	75	−1577	−636	−2847
5	2.870	85	−803	−134	−1975
6	2.366	95	−921	−84	−2681
Mean ± SEM					−2260 ± 409

Calorimetrically determined enthalpy, ΔH, associated with relaxation of covalently closed circular superhelical ColE1 *amp* plasmid DNA in the presence of topoisomerase at 37°C in 0.01 M Tris/0.002 M $MgCl_2$/0.001 M EDTA, pH = 8.0. *M* of ColE1 *amp*, 7.2×10^6; ΔQ, heat change.

lysis of acyclic phosphate esters (42). Summarizing the enthalpy determinations, one can say that relaxation of superhelical turns of ColE1 *amp* DNA is a strongly exothermic reaction. Therefore, introduction of superhelicity is endothermic.

Gibbs Free Energy of Supercoiling. Several quantitative treatments of the Gibbs free energy of superhelix formation have been given (23, 25, 33, 34), which all resulted in approximately quadratic dependence of the Gibbs free energy on the titratable superhelix density σ given in Eq. 1 (2):

$$\Delta G_\sigma \simeq 10\, RTN\sigma^2, \qquad [1]$$

in which N refers to the number of base pairs, R is the gas constant, and T is the absolute temperature. The factor 10 stems from the results in ref. 33. We assumed that the value of 1000 for the expression $N{\cdot}K/RT$ in Depew and Wang's treatment determined for PM2 DNA is also valid for ColE1 *amp* plasmid DNA, which has a 10% larger number of base pairs (11,000).

We obtained values for the superhelix density applying two independent methods. We used conventional CsCl density gradient centrifugation with PM2 and fd DNA as standards and used the fluorescence assay as described by Lee and Morgan (41) in the presence of a topoisomerase. As distinguished from ref. 41, we used for relaxation the ω protein, which can only relax negatively supercoiled DNA but not positively supercoiled molecules. This difference does not, however, affect the accuracy of the procedure.

The average value obtained for the superhelix density of ColE1 *amp* DNA at 37°C is $\sigma = -0.057 \pm 0.004$. This value corresponds to a Gibbs free energy of ΔG_σ (37°C) = 921 ± 130 kJ·(mol of plasmid)$^{-1}$. Using the fundamental thermodynamic relationship $\Delta G = \Delta H - T\Delta S$, one obtains for the entropy change on superhelix formation (4.3 ± 1.8) kJ·(mol of plasmid·K)$^{-1}$. The error limits given for ΔS refer to the combinations of the lower-limit ΔH value with the upper-limit ΔG value and vice versa.

Calculation of DNA Rigidity. Although much is known about long-range segmental motions in DNA, there is relatively little information about local motions in the DNA helix. Parameters that describe the stability of DNA against torsion and bending are the torsional and flexural rigidity. These parameters are important for understanding the structural features of duplex DNA such as supercoiling and packaging in chromosomes and bacteriophage heads, which can be qualitatively accounted for by models based on the elastic properties of DNA (43–47). Various estimates of the torsional rigidity, C, have been published; the largest collection of data is found in ref. 48. Values of the torsional rigidity have been obtained from time-dependent fluorescence depolarization measurements on ethidium intercalation in linear and circular DNAs (48–50) from EPR spectroscopy of intercalated spin probes (51, 52) and from the free energy of supercoiling (33, 34, 53). There is a considerable spread in the data, even if one compares only the few values published for the torsional rigidities of circular DNA. The C values, which can be calculated from the ΔG data of Depew and Wang (33) and Pulleyblank *et al.* (34) for several circular DNAs, range from $C = 0.64 \times 10^{-19}$ to $C = 1.086 \times 10^{-19}$ erg·cm (1 erg = 0.1 μJ), whereas the C value of pBR322 derived from the fluorescence depolarization measurements by Millar *et al.* (48) is 1.95×10^{-19} erg·cm.

It does not appear very likely that these large differences in the torsional rigidities originate from experimental errors; rather, a systematic error appears to be responsible for the fact that consistently smaller values are determined for C when Gibbs energy values are used in the calculations. It has been observed by Millar *et al.* (48) that the torsional rigidities of polynucleotides correlate without exception with the calculated stacking energies and not with the t_m (melting temperatures of double-stranded DNA) values of the systems. Although relative t_m values do not correspond rigorously to relative stabilities, which are determined by ΔG, they usually constitute a fair approximation.

We assume on the basis of this observation that not the Gibbs energy of supercoiling but rather the enthalpy of the reaction may be the appropriate quantity for the calculation of torsional rigidities—at least to compare the numerical values derived from fluorescence depolarization measurements of intercalated dyes. Furthermore, if one assumes that the enthalpy of supercoiling results exclusively from torsion and not from bending as was done in the majority of studies cited above, one can relate the observed enthalpy to torsional rigidity by the expression for the torsional elastic energy U (49)

$$U = \frac{C}{2}\psi^2/l,$$

in which U is the energy of a twist ψ in a length l. Assuming $l = 3.4 \times 10^{-8}$ cm for the distance of two base pairs in the B-DNA structure, we obtain for the twist in this length $\psi = 2\pi\tau/N$, with τ denoting the number of superhelical turns and N being the number of base pairs in the plasmid. τ is calculated from the superhelical density by multiplying with 1/10th of the number of base pairs. Using the enthalpy value of 2260 kJ·(mol of plasmid)$^{-1}$, one obtains for the torsional rigidity of ColE1 *amp* plasmid DNA $C = (1.79 \pm 0.3) \times 10^{-19}$ erg·cm, the error limits referring to calculations with $\Delta H = 2669$ and $\Delta H = 1851$ kJ·(mol of plasmid)$^{-1}$, respectively.

DISCUSSION

The present study on the thermodynamics of superhelix formation provides directly measured enthalpy values, which permit an unambiguous assignment of the enthalpic and entropic contributions to the unfavorable Gibbs free energy of supercoiling. The large positive enthalpy concomitant with introducing superhelical turns is clearly the major factor determining the unfavorable ΔG of supercoiling. Assuming that ColE1 *amp* plasmid is not exceptional in its thermodynamic properties, the present result suggests that, in general, supercoiling of DNA is enthalpy-determined. This conclusion is supported by measurements on PM2 DNA performed in our laboratory, where preliminary data indicate that also for PM2 DNA the positive enthalpy involved in superhelix formation is the dominant component of the superhelix free energy. These results resolve the ambiguity in the interpretation of previous measurements (33, 34) on the thermodynamics of supercoiling.

In a recent publication on the unwinding of double-stranded linear DNA by dehydration and subsequent closing by ligase, estimates of the enthalpy of unwinding one link of the DNA helix were given (54). A ΔH value of 51 ± 1.7 kJ·(mol of 10 base pairs)$^{-1}$ was reported, and it was considered to be independent of both DNA size and the linking number. These ΔH values should be roughly comparable to the enthalpy associated with introducing one superhelical turn. The superhelix density of 0.057 determined in this study for ColE1 *amp* DNA is equivalent to 63 superhelical turns. Using this value, we obtain 34.9 kJ·(mol of superhelical turns)$^{-1}$, which is approximately 70% of the ΔH reported by Lee *et al.* (54). In view of the vastly different methods used, the agreement appears to be relatively good.

Elastic Properties of DNA. The torsional rigidity of ColE1 *amp* DNA has been calculated on the assumption that only torsion contributed to the enthalpy of supercoiling, the bending contribution being negligible. This assumption certainly constitutes only an approximation. It is due to a general un-

certainty of how to partition unambiguously the overall experimental data into contributions from bending and torsion. Therefore, in the majority of studies (33, 34, 50, 51, 52), only the limiting case of vanishing bending contributions has been considered. Thomas *et al.* (50) derived a value of $C = 1.29 \times 10^{-19}$ erg·cm from time-dependent fluorescence depolarization measurements on ethidium intercalated in viral ϕ29 DNA. Hurley *et al.* (51) determined $C = 1.2 \times 10^{-19}$ erg·cm by EPR studies on spin probes intercalated in chicken erythrocyte DNA. Barkley and Zimm (49) obtained a value of $C = 4.125 \times 10^{-19}$ erg·cm from fitting the fluorescence depolarization data of Wahl *et al.* (55) to their theoretical model. The previously mentioned torsional rigidities for superhelical DNA calculated from the ΔG data of Pulleyblank *et al.* (34) and Depew and Wang (33) range from $0.64 \cdot 10^{-19}$ erg·cm to $1.086 \cdot 10^{-19}$ erg·cm. Just as all C values mentioned so far, they have been calculated on the assumption that only torsion contributed to the Gibbs energy of superhelix formation. The elastic model of DNA developed by Barkley and Zimm (49) permits one to analyze fluorescence depolarization data in terms of bending and twisting contributions, provided the time resolution of the measurements is high enough. Millar *et al.* (48) were able to report data on the torsional rigidity of calf thymus DNA ($C = 1.43 \times 10^{-19}$ erg·cm) and circular pBR322 DNA ($C = 1.95 \times 10^{-19}$ erg·cm), where they took the flexural rigidity into account. Their value for calf thymus DNA is higher [with the exception of the value by Barkley and Zimm (49)] than all other torsional rigidities for linear DNAs, and the value for superhelical pBR322 DNA is decidedly higher than that for linear DNA. Millar *et al.* (48) emphasize that, for a correct analysis of the fluorescence decay measurements, inclusion of the bending dynamics is very important. Therefore, it is surprising that the torsional rigidity obtained for circular ColE1 *amp* DNA ($C = 1.79 \times 10^{-19}$ erg·cm) in the present study from the enthalpy of supercoiling is in good agreement with their value obtained for pBR322 because bending contributions to ΔH have been neglected in our calculation. We do not believe that the agreement is only fortuitous. It may well be that the fast local bending motions of DNA in the picosecond and nanosecond ranges, responsible for the fluorescence anisotropy decay of the intercalated dye, do not contribute significantly to the reaction enthalpy associated with the transformation of underwound, supercoiled circular DNA into relaxed circular DNA. This assumption is reasonable in view of the large size of the plasmid and the moderate superhelix density.

Recently Vologodskii *et al.* (53) performed Monte Carlo calculations of the bending contributions to the linking number distribution of supercoiled DNAs, which resulted in a value of $C = (1.65 \pm 0.33) \times 10^{-19}$ erg·cm. However, these calculations also may be useful for solving the problem of the relative contributions of bending and torsion. The calculations demonstrated that the width of the writhing-number distribution equals approximately half the width of the linking-number distribution as determined in refs. 33 and 34; in other words, half of the total number of titratable superhelical turns is realized as writhing of the helix axis, and the other half, as axial twisting. Application of this result to energy data is not straightforward. The number of superhelical turns is not necessarily equal to the value of the writhing number (56), and it is not obvious whether the results of Vologodskii *et al.* (53), which are valid for the Gibbs energies, can be assumed to apply to the reaction enthalpies. However, when making these assumptions, only half of the overall ΔH—i.e., about 1100 kJ·(mol of plasmid)$^{-1}$—would be attributed to torsional energy; the residual enthalpy would be assigned to bending. By identifying the writhing number with the number of tertiary turns as a first approximation, only half of the titratable superhelical density is realized as twist, according to ref. 53. Because the torsional energy depends on the square of the twist, the reduction by a factor of 2 of both the enthalpy and the twist does not cancel each other. The calculation leads to a significantly larger value for the torsional rigidity C of 3.58×10^{-19} erg·cm.

In view of the differences between rigidity values obtained by different experimental approaches and on the basis of different theoretical models, much experimental and theoretical work appears to be necessary for resolution of the problems.

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