Energy Coupling in DNA Gyrase: A Thermodynamic Limit to the Extent of DNA Supercoiling†

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ABSTRACT: ATPαS (R_p) has been shown to support the supercoiling of plasmid pBR322 catalyzed by *Escherichia coli* DNA gyrase at comparable rates to the natural substrate ATP and is able to promote the introduction of one more superhelical turn than ATP. The difference in free energy change between consecutive rounds of supercoiling in gyrase-mediated reactions is calculated to be 2.6 kJ mol^{-1}. The difference in free energy of hydrolysis of ATP and ATPαS (R_p) has been determined from the difference in the equilibrium constants for the phosphorylation of arginine established by arginine kinase. This equilibrium constant has been found to be displaced by a factor of about 1.5, corresponding to a greater free energy of hydrolysis of ATPαS (R_p) compared to ATP of approximately 1 kJ mol^{-1}. This difference in free energy can be tentatively ascribed to a relative destabilization of the MgATPαS (R_p) complex with respect to MgATP. Assuming that the stoichiometry of the coupled reactions requires two ATPs hydrolyzed per round of supercoiling, ATPαS (R_p) should be capable of providing an additional ca. 2 kJ mol^{-1} of free energy for DNA supercoiling, which is in good agreement with estimates for the additional free energy required to achieve a further round of supercoiling. These results provide direct evidence to support the proposal that the extent of DNA supercoiling by DNA gyrase is limited by the free energy of hydrolysis of the nucleotide.

DNA gyrase (E.C.5.99.1.3) is a bacterial type-II topoisomerase which catalyzes the energetically unfavorable negative supercoiling of DNA by coupling this reaction to the hydrolysis of ATP (Wang, 1985; Reece & Maxwell, 1991). While all topoisomerase are able to relax negatively supercoiled DNA, gyrase is unique in its supercoiling activity. The active enzyme from *Escherichia coli* consists of two proteins (A and B) of M_r 97K and 90K, respectively, organized as an A_2B_2 complex. In addition to the DNA supercoiling reaction, gyrase can also catalyze the relaxation of both positively and negatively supercoiled DNA, the knotting and unknotting of DNA, and the catenation and decatenation of duplex circles.

Although details of the DNA supercoiling reaction mechanism are not fully understood, the principal steps have been identified (Maxwell & Gellert, 1986; Reece & Maxwell, 1991). Gyrase binds to DNA, and a complex is formed in which a DNA segment of approximately 120 bp becomes wrapped around the protein core in a positive superhelical sense. The enzyme cleaves the DNA in both strands via a nucleophilic mechanism in which the 5′-phosphoryl groups become covalently attached to tyrosines at position 122 of the A subunits. Another section of the DNA is then translocated through this double-strand break leading to a reduction in the linking number of 2 (i.e., the introduction of two negative supercoils) following reversal of the strand cleavage. The driving force for this energetically unfavorable reaction is provided by the concomitant hydrolysis of ATP; it is thought that two molecules of ATP are hydrolyzed per supercoiling cycle. The usual DNA substrates used in studies of supercoiling by gyrase are duplex circles of several thousand base pairs. With such substrates, the limiting specific linking difference (superhelical density; σ) achievable by gyrase has been shown to be –0.11 (Westerhoff et al., 1988; Bates & Maxwell, 1989). The estimate of the free energy required to reach this limit corresponds to the likely free energy available from hydrolysis of two ATPs (Maxwell & Gellert, 1986; Westerhoff et al., 1988; Bates & Maxwell, 1989). Gyrase has also been shown to interact with considerably smaller circles as well as short linear DNA sequences (Bates & Maxwell, 1989; Maxwell & Gellert, 1984). The lower limit in the size of circle that can be supercoiled is 174 bp, for which two alternative explanations can be offered (Bates & Maxwell, 1989): (i) The free energy required to carry out a cycle of supercoiling in circles below this size limit exceeds that available from the hydrolysis of ATP (a thermodynamic limit). (ii) The size of the smaller circles is insufficient to allow the necessary complexation and conformational changes required through the catalytic cycle (a steric limit). The latter explanation seems less likely since it has been shown that gyrase can relax positively supercoiled circles considerably smaller than 174 bp (i.e., 116–152 bp) in a nucleotide-dependent reaction mechanistically analogous to negative supercoiling. The limiting specific linking difference for supercoiling of small DNA circles has been shown to be the same as that determined for large circles within the limits of the methods of determination (σ = –0.11), which may favor a thermodynamic limit to the extent of supercoiling (Bates & Maxwell, 1989). In this study we have used analogues of ATP to probe the energy coupling of the gyrase-catalyzed reaction.

EXPERIMENTAL PROCEDURES

DNA Preparation. Native supercoiled pBR322 was isolated from *E. coli* cells by alkaline lysis (Birnboim & Doly, 1979), followed by two cycles of cesium chloride centrifugation. Relaxed pBR322 was prepared as previously described (Bates & Maxwell, 1989) and purified by cesium chloride centrifugation.

Protein Purification. E. coli DNA gyrase A and B subunits were prepared by two methods: Mizuuchi et al. (1984) (gift

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from Dr. J. Tamura, NIH) and Hallett et al. (1990); the enzyme from either source gave identical results. The A_2B_2 active enzyme was reconstituted from the subunits as previously described (Bates & Maxwell, 1989). Chicken erythrocyte DNA topoisomerase I was prepared by the method of Trask and Muller (1984), omitting the final phenyl-Sepharose purification step.

Arginine kinase and pyruvate kinase/lactate dehydrogenase mix (PK/LDH) were from Sigma. Arginine kinase was dialyzed into 200 mM Hepes, pH 8.0, 5 mM DTT, and 100 mM glycine and stored at 4 °C.

Synthesis and Purification of Nucleotides. A racemic mixture of ATPαS was synthesized according to the method of Ludwig and Eckstein (1989). The crude product was purified by chromatography on a DEAE–Sephadex A25 column (1.6 cm × 80 cm) eluting with a linear gradient of 1 L each of 0.1–1 M TEAB (triethylammonium bicarbonate), pH 7.5. ATPαS was identified by ^{31}P NMR. The R_p and S_p diastereoisomers were separated preparatively by reverse-phase HPLC using a Hewlett-Packard HP1090 dual pump system with an automatic injector and a C_{18} reverse-phase column (10 mm × 25 mm) packed with ODS Hypersil (5 μm, from FSA Chromatography). Repeated runs were eluted with 150 mM TEAB, pH 7.5, containing a linear gradient of acetonitrile from 0 to 20% in 30 min at a flow rate of 4 mL/min. The purity of the diastereoisomers was verified by analytical HPLC. ATP was obtained from Sigma as the disodium salt. A sample was purified and converted to the triethylammonium salt by chromatography on a DEAE–Sephadex A25 column as described above for ATPαS. Both salts of ATP behaved identically in the enzyme reactions. Concentrations of adenine nucleotides were determined by their absorbance at 259 nm assuming $\epsilon = 15.4$ cm^{-1} mM^{-1}.

DNA Gyrase Reactions. Relaxed pBR322 DNA (10 μg/mL) was incubated with DNA gyrase (2.0 nM) in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 5 mM $MgCl_2$, 1.8 mM spermidine, 9 μg/mL tRNA, 5 mM DTT, 1 mM nucleotide, 0.36 mg/mL BSA, and 6.5% (w/v) glycerol at 25 °C. At various times 30-μL aliquots were removed, and the reactions were stopped by addition of 10 μL of 40% sucrose, 100 mM Tris-HCl, pH 7.5, 100 mM EDTA, and 0.5 mg/mL bromophenol blue and extraction with an equal volume of chloroform/isoamyl alcohol (24:1). The DNA was then analyzed by electrophoresis through 0.8% agarose gels in 40 mM Tris base, 30 mM sodium dihydrogen phosphate, and 0.5 mM EDTA in the presence of various concentrations of chloroquine. Linking numbers of topoisomers were assigned by the method of Keller (1975).

Measurement of Equilibrium Concentration of Nucleotides in Arginine Kinase Reaction. Arginine solutions were quantitated by a PK/LDH coupled assay. The reaction mixture contained 200 mM Hepes, pH 8.0, 10 mM magnesium acetate, 100 mM potassium chloride, 5 mM DTT, 250 μM NADH, 400 μM PEP, 75 units pyruvate kinase, 90 units lactate dehydrogenase, 1 mM ATP, arginine kinase (0.165 mg/mL; 120 units/mg), and arginine. Two methods were used to determine the equilibrium constants for the oxynucleotide and thionucleotide reactions catalyzed by arginine kinase.

(*i*) *^{31}P NMR Method.* (Lerman & Cohn, 1980; Lee & O'Sullivan, 1985). Reactions were carried out in 5-mm NMR tubes positioned inside 10-mm NMR tubes containing D_2O and comprised the following: 200 mM Hepes, 5 mM DTT, 40 mM magnesium acetate, 30 mM arginine, 30 mM nucleotide, and arginine kinase (1.65 mg/mL; 120 units/mg) in a total volume of 500 μL. Spectra were obtained at 121.5 MHz in Fourier transform mode on a Bruker AM300 spectrometer with the sample temperature controlled at 30 °C. Spectral parameters were as follows: spectral width, 3600 Hz; pulse width, 10 ms; aquisition time, 1.1 s; pulse delay, 10 s; line broadening, 1.7 Hz.

(*ii*) *HPLC Method.* The arginine kinase reactions contained 200 mM Hepes, 5 mM magnesium acetate, 1 mM arginine, 1 mM nucleotide, and 10 μL arginine kinase (1.65 mg/mL; 120 units/mg) in a total volume of 500 μL. (The reactions with 30 mM nucleotide were identical except that 40 mM magnesium acetate, 30 mM arginine, and 50 μL arginine kinase were used.) Samples (100 μL) were removed after 30 min, 2 h, and 12 h, and 25 μL of a 2% solution of SDS followed by 12 μL of 10 mg/mL proteinase K were added. The samples were incubated at 30 °C for 30 min and then quick frozen and stored at −70 °C until analysis of HPLC. The samples (26 μL) were loaded directly onto a C_{18} reverse-phase analytical column, fitted with a C_{18} guard column, and eluted with a linear gradient of acetonitrile (0–10%, in 25 min; flow rate 1.5 mL/min) in 100 mM TEAB, pH 7.6. The relative concentrations of di- and trinucleotides were determined from the integrals of their peaks in the chromatograms.

RESULTS AND DISCUSSION

Extent of Supercoiling. The relaxed form of plasmid pBR322 (either obtained by the action of a type-I topoisomerase or by the ligation of a linear or nicked precursor) consists of a Gaussian distribution of topoisomers with individual linking numbers differing by one, which can, under appropriate conditions, be distinguished by gel electrophoresis (Pulleyblank et al., 1975; Depew & Wang, 1975). Given sufficient ATP and time of incubation, gyrase catalyzes the change in linking number leading to an average limiting value of the specific linking difference ($\sigma = -0.11$; Westerhoff et al., 1988; Bates & Maxwell, 1989). It has been previously suggested that the limit for the extent of supercoiling is determined by the free energy of hydrolysis of ATP (Maxwell & Gellert, 1986; Westerhoff et al., 1988; Bates & Maxwell, 1989); the free energy required to introduce the final two superhelical turns in pBR322 has been estimated to be ca. 116 kJ mol^{-1}, which is comparable to the free energy available from the hydrolysis of two ATPs (ca. 120 kJ mol^{-1}) (Simmons & Hill, 1976). Furthermore, the **difference** in energy change between consecutive steps in the gyrase supercoiling reaction can be shown to be 2.6 kJ mol^{-1} (Figure 1), based on the data of Horowitz and Wang (1984). The energy required for supercoiling can only be regarded as approximate since a number of assumptions have to be made. Experimentally, the calculation of the free energy of supercoiling has been based on either analysis of the Gaussian distributions of topoisomers observed by gel electrophoresis (Depew & Wang, 1975; Pulleybank et al., 1975; Shore & Baldwin, 1983; Horowitz & Wang, 1984) or on the binding isotherms of ethidium bromide to the DNA (Bauer & Vinograd, 1970; Hsieh & Wang, 1975). Both of these methods have analyzed DNA at levels of supercoiling below those pertaining to this study, and the reported dependence of the free energy of supercoiling on the square of the linking difference may break down at higher specific linking differences (σ).

If the limit to supercoiling is thermodynamic, then gyrase will continue to introduce superhelical turns into pBR322 until the energy for the next round exceeds that available from the hydrolysis of two ATPs. One obvious way to determine whether the extent of supercoiling is limited by the free energy of hydrolysis of the nucleotide would be to employ a nucleotide

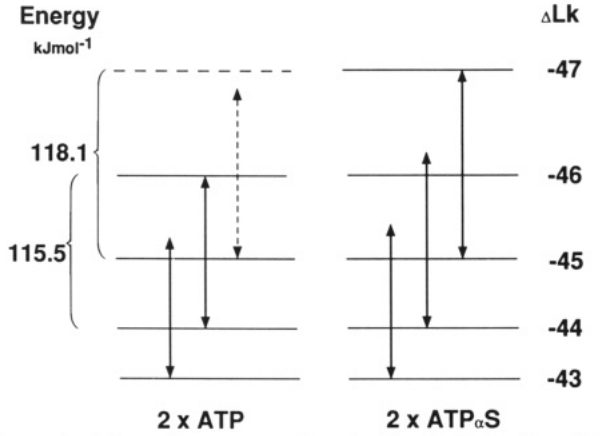
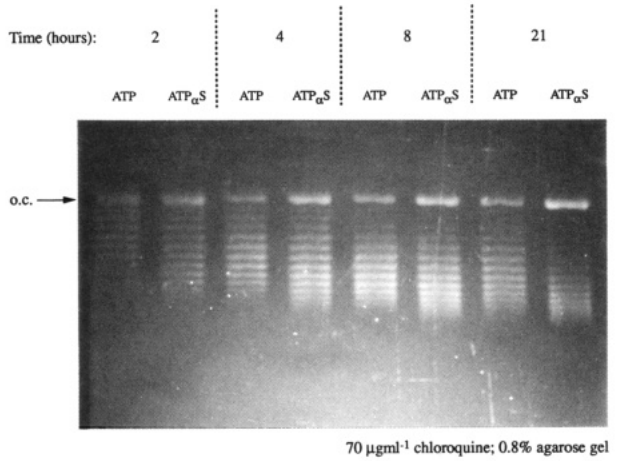


FIGURE 1: Schematic representation showing the limit of specific linking difference promoted by ATP and ATPαS. For the purposes of illustration, the energy separations are not drawn to scale in order to emphasize the origin of the difference between the two nucleotides. It should be noted that for consecutive steps in the gyrase supercoiling reaction (i.e., a ΔLk of n to $n-2$ and $n-1$ to $n-3$) the difference in free energy change ($\Delta\Delta G$) between these steps can be shown to be: $\Delta\Delta G = K[(n-3)^2 - (n-1)^2] - [(n-2)^2 - n^2]\} = 4K$, where K is the elastic constant for the dependence of supercoiling free energy on linking difference. Therefore, for pBR322: $\Delta\Delta G = 4(1130RT/4361) = 2.57$ kJ mol^{-1} (at 25 °C). As shown, this estimate holds for any pair of consecutive steps in supercoiling regardless of the absolute value of the specific linking difference.



70 μgml^{-1} chloroquine; 0.8% agarose gel

FIGURE 2: Extent of supercoiling with ATP and ATPαS (R_p). Gyrase concentration was 2 nM; DNA concentration was 3.4 nM; nucleotide concentration was 1 mM. The figure shows the later time-points of the supercoiling reactions where the specific linking difference of the DNA is approaching a maximum. At apparent equilibrium, the topoisomer of greatest linking difference corresponded to a ΔLk of –46 in the ATP reaction and –47 in the ATPαS reaction.

Table I

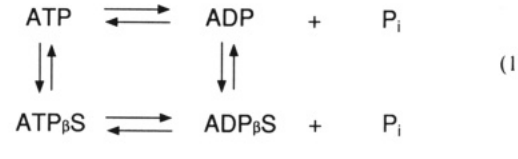
nucleotide	pK_a's	
ATP	6.63 ± 0.04[a]	6.7[b]
ADP	6.66 ± 0.01[a]	6.8[b]
MgATP	4.72 ± 0.05[a]	5.3[b]
MgADP	5.46 ± 0.06[a]	
ATPαS	6.65 ± 0.01[a]	
ADPαS	6.77 ± 0.08[a]	
AgATPαS	5.12 ± 0.03[a]	
MgADPαS	5.27 ± 0.03[a]	
ATPβS	6.64 ± 0.10[a]	6.5[b]
ADPβS		5.2[b]

[a] Determined by Pecoraro et al. (1984) from ^{31}P NMR. Values refer to the S_p isomers of ADPαS and ATPαS and the (R_p) isomer of ATPβS. [b] Determined by Jaffe and Cohn (1978) from ^{31}P NMR. Values refer to the S_p isomer of ATPβS.

analogue with greater free energy of hydrolysis than ATP itself and establish whether this is able to push the supercoiling reaction further. It has been shown previously that the free energy of hydrolysis of ATPβS is approximately 10 kJ mol^{-1} more exergonic than ATP (Jaffe & Cohn, 1980; Lerman & Cohn, 1980). This in part must arise from the fact that thiophosphates are stronger acids than related phosphates, such that ADPβS^{3-} is significantly more thermodynamically stable than ADP^{3-} and also a better leaving group (Frey, 1989). Based on comparison of ATPγS with ATP and of ADPβS with ADP, it has been shown that the pK_a of the terminal phosphate is shifted ca 1.5 units lower on substituting one of the oxygens with sulfur. Cohn and co-workers have used the greater free energy of hydrolysis of ATPβS to demonstrate that the magnitude of the transmembrane Ca^{2+} gradient in sarcoplasmic reticulum is not limited by the free energy of hydrolysis of the nucleotide (Pintado et al., 1982). Unfortunately attempts to use a similar approach to study the energy coupling in DNA gyrase failed because ATPβS is not able to support efficient supercoiling (Weiner, Maxwell, and Cullis, manuscript in preparation). It appears that DNA gyrase catalyzes the hydrolysis of ATPβS but that this is only poorly coupled to DNA supercoiling. As part of a study of other thionucleotide analogues of ATP with DNA gyrase we have observed that ATPαS (R_p) is as good a substrate for DNA gyrase as ATP, both in terms of the ATPase and DNA supercoiling reactions. Interestingly, we have established that ATPαS (R_p) is able consistently to introduce one additional superhelical turn (Figure 2). ATP was found to support a maximum ΔLk of –46 compared to a value of –47 for ATPαS. The specific linking difference of –0.11 with ATP is the same as that found previously (Westerhoff et al., 1988; Bates & Maxwell, 1989).

Estimation of Free Energy Differences. If the assumption is made that the ionization of nucleotides is a measure of the relative stabilities of these species, an estimate of the relative free energies for hydrolysis of the various nucleotides on the basis of the differences in pK_a's can be made. Implicit in this approach is the assumption that the differences in the pK_a's arises principally from the difference in thermodynamic stabilities of the fully ionized nucleoside polyphosphates. Although this may not seem to be justified, the strong electrostatic interaction between the adjacent negative charges in the *gem*-dianion, which is generated in the last ionization, together with the likely strong interaction of the fully ionized species with solvent may be dominant factors that justify this assumption.

We have applied this approach first to the case of ATPβS for which the free energy of hydrolysis relative to ATP is known. Consider the hypothetical thermodynamic cycle shown below:



(1)

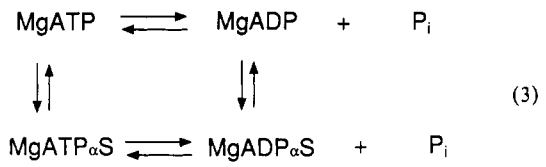
The hypothetical equilibria between ATP and ATPβS and between ADP and ADPβS represent the relative stabilities of these species to be estimated from the differences in pK_a's. The literature pK_a values are shown in Table I. Within experimental error for ATP and ATPβS the ΔpK_a is zero,

whereas for ADP and ADPβS the ΔpK_a is 1.6. This can be converted into an energy term using eq 2:

$$\Delta G = -RT \ln [K_{thio}/K_{oxy}] \quad (2)$$

where $[K_{thio}/K_{oxy}]$ is the antilog$_{10}$ of the ΔpK_a. Because of the nature of a thermodynamic cycle, the ratio of equilibrium constants for these two hypothetical equilibria must be the same as the ratio of equilibrium constants for the two hydrolysis reactions. The free energy difference calculated in this way is 9.3 kJ mol^{-1}, which is in satisfactory agreement with the published measured value of ca. 10 kJ mol^{-1} (Jaffe & Cohn, 1980; Lerman & Cohn, 1980). If valid, this approach would imply that the more favorable free energy of hydrolysis of ATPβS arises almost completely from the greater thermodynamic stability of ADPβS as compared to ADP, which seems intuitively correct.

Applying this approach to MgATPαS and MgATP we have to consider the hypothetical thermodynamic cycle shown below:



(3)

From the pK_a values shown in Table I it can be seen that ATP and ATPαS have pK_a's that are the same within experimental error and that ADP has a slightly lower pK_a than ADPαS. This would lead to the conclusion that if anything ATP should have a marginally greater free energy of hydrolysis than ATPαS. However, the supercoiling reactions are all conducted in the presence of excess magnesium and at a concentration where >95% of the nucleotides are complexed. The correct pK_a's to use are therefore those for the nucleotide–Mg complexes. From Table I the ΔpK_a for MgADPαS and MgADP is 0.19, with the thiophosphate being more stable. For MgATP and MgATPαS the ΔpK_a is 0.4, but this time the thiophosphate is the least stable. Combining these ΔpK_a's and conversion to a free energy term using eq 2 gives a value of 3.4 kJ mol^{-1} as an upper estimate for the difference in free energy of hydrolysis of ATP and ATPαS, which would be sufficient to account for the additional supercoiling. Since the higher pK_a of MgATPαS is the dominant factor, it would appear that the more favorable free energy of hydrolysis of ATPαS is likely to arise from the relative destabilization of the MgATPαS with respect to MgATP. It should be noted that account could not be taken of the presence of magnesium for ATPβS because of the lack of a pK_a for MgADPβS in the literature. However, in this case the differences in pK_a's are much larger and the effects of Mg will presumably be less significant.

The above analysis is almost certainly an oversimplification which takes into account only differences in pK_a's. The study of Lawson and Veech (1979) on the effects of pH and free Mg^{2+} on the creatine kinase reaction showed that the equilibrium constant for this reaction varied with the concentration of free Mg^{2+}, as did the equilibrium constants for a number of other phosphotransferase reactions of biological importance. Since the stability constants for MgATP and MgADP complexes are different from the corresponding thionucleotide complexes (Peccoraro et al., 1984), the effect of free Mg^{2+} may also contribute to the differences in free energy of hydrolysis between ATP and ATPαS and ATPβS. However, despite the simplicity of the above analysis, the calculated value for the free energy of hydrolysis of ATPβS is in excellent agreement with the experimentally determined value (Jaffe & Cohn, 1980; Lerman & Cohn, 1980), and the calculation for ATPαS served to prompt the experimental measurements discussed below.

Table II

$$\text{ATP + arginine} \overset{Mg^{2+}}{\rightleftharpoons} \text{ADP + phosphoarginine}$$

K^a	K_{thio}/K_{oxy}	$\Delta\Delta G$ (kJ mol^{-1})
$K_{ATP} = 0.25 \pm 0.1$		
$K_{ATP\alpha S} = 0.36 \pm 0.2$	1.44 ± 0.14	0.92 ± 0.2
$K_{ATP\beta S} = 27.8 \pm 4$	111 ± 21	11.8 ± 0.5

[a] The equilibrium constants K_{oxy} and K_{thio} reported here are apparent values and are not equal to the thermodynamic equilibrium constants because the hydrogen ion released in the reaction has been neglected.

Measurement of Displaced Equilibrium of ATPαS with Arginine Kinase. Because of the relatively small energy difference inferred from the supercoiling reaction together with the uncertainties concerning the assumptions inherent in the above calculations, we have determined the displacement of the equilibrium between the various nucleotides and arginine/phosphoarginine to measure directly the difference in free energy of hydrolysis between ATP and ATPαS, an approach first used by Lerman and Cohn (1980) for ATPβS. We have elected to use an HPLC method in which the equilibrium mixture is rapidly denatured by addition of SDS and proteinase K (see Experimental Procedures). Control experiments have shown that this leads to rapid and complete irreversible inactivation of the enzyme, and furthermore, an equilibrium established for ATP and arginine in the presence of arginine kinase determined simultaneously by this method and the ^{31}P NMR method described by Lerman and Cohn (1980) were the same within experimental error. One advantage of the HPLC method is that the equilibria can be measured under conditions analogous to those for the supercoiling reaction (1 mM nucleotide; 5 mM Mg; etc.). The concentrations of the various nucleotides were determined by integration of the HPLC peaks, and the initial concentration of arginine was determined enzymatically. Confirmation that the equilibrium had been established was shown by analyzing samples taken at various times. The equilibria between arginine and either ATP, ATPαS, or ATPβS determined by HPLC are shown in Table II. Each value is an average of three separate HPLC runs of samples taken at various time points. The absolute values for the equilibrium constants for ATP and ATPβS differ somewhat from those previously reported (Lerman & Cohn, 1980) but, given the good agreement between the HPLC and ^{31}P NMR based methods under identical conditions (see above), the differences in the values in Table II probably reflect the different experimental conditions. The major differences are the 30-fold lower concentration of nucleotide and the 5-fold excess of magnesium ion used in this study, since these conditions represent those used for the gyrase assays. It should be appreciated that these are only apparent equilibrium constants since the hydrogen ion released in the reactions has been neglected, consequently these values will be strongly pH dependent. The determination of the difference in free energy of hydrolysis between ATP and ATPβS using the values reported here gives a value of 11.8 kJ mol^{-1} in favor of ATPβS as compared to the literature value of 10.5 kJ mol^{-1}.

For ATPαS (R_p) the equilibrium with arginine established by arginine kinase is displaced, compared to that for ATP, by a factor of approximately 1.5 in favor of ADPαS and P_i. The

difference is considerably smaller than that for ATPβS in keeping with expectation, but is entirely reproducible. This difference means that the free energy of hydrolysis of ATPαS (R_p) is more exergonic than ATP by 0.92 kJ mol^{-1}. Furthermore, a study on phosphomevalonate kinase (Lee & O'Sullivan, 1985) has also reported a shift in the equilibrium position with ATPαS (S_p) as compared to ATP of 1.95 (K_{thio}/K_{oxy}), which corresponds to an estimated difference in free energy of hydrolysis of ATPαS of 1.7 kJ mol^{-1}. The difference between these two values may reflect the different conditions and the fact that opposite diastereoisomers have been used.

Energy Coupling in DNA Gyrase. The extent of supercoiling of pBR322 catalyzed by DNA gyrase in the presence of ATPαS is greater than with ATP by a linking difference of one. The extent of supercoiling is therefore directly associated with the nucleotide in some manner. Because of earlier suggestions that the limit to supercoiling may be associated with the free energy of hydrolysis of the nucleotide (a thermodynamic limit), we have looked to see whether the additional round of supercoiling promoted by ATPαS (R_p) may be attributed to greater free energy of hydrolysis as compared to ATP. As shown above, there is a small but measurable difference in free energy of hydrolysis as gauged from the arginine kinase reaction equilibria. We have determined the difference in free energy of hydrolysis of ATPαS (R_p) and ATP to be approximately 1 kJ mol^{-1}. This estimate has the merit that it has been determined under conditions close to those in the gyrase supercoiling reaction, and furthermore, both gyrase and arginine kinase (Cohn et al., 1982) handle the (R_p) diastereoisomer of ATPαS. Gyrase has two ATP binding sites, one associated with each B subunit (Tamura & Gellert, 1990; Wigley et al., 1991), and two ATPs are thought to be hydrolyzed by gyrase per round of supercoiling (Sugino & Cozzarelli, 1980). Furthermore, this hydrolysis is believed to be a cooperative process (Maxwell et al., 1986; Tamura et al., 1992). Thus it appears that the hydrolysis of two nucleotides is coupled to each cycle of DNA supercoiling. This being the case ATPαS (R_p) will provide approximately 2 kJ mol^{-1} additional free energy for supercoiling as compared to ATP. There is good agreement between this value and the calculated difference in energy change between consecutive rounds in supercoiling. These results are in accord with the proposal that supercoiling is limited by the free energy of hydrolysis of the nucleotide. However, the uncertainties concerning estimates for the differences in energy for consecutive steps in supercoiling should not be overlooked. These estimates arise from calculations based on plasmids with low specific linking differences, and extrapolation to high specific linking differences may not be valid if alterations of the DNA conformation occur. The agreement could also be fortuitous and the additional round of supercoiling may arise from other properties of the thionucleotide. Our observation that the K_m^{app} for ATPαS (R_p) is ca. 10-fold tighter than for ATP (Weiner, Maxwell, and Cullis, manuscript in preparation) may provide an alternative explanation for the additional supercoiling, but in view of the fact that gyrase does not appear to obey Michaelis–Menten kinetics (Maxwell et al., 1986; Tamura et al., 1992), interpretation of these apparent K_m's should be viewed with caution. Thus, the simplest interpretation of these observations is that the extent of supercoiling is directly determined by the free energy of hydrolysis of the nucleotide.

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