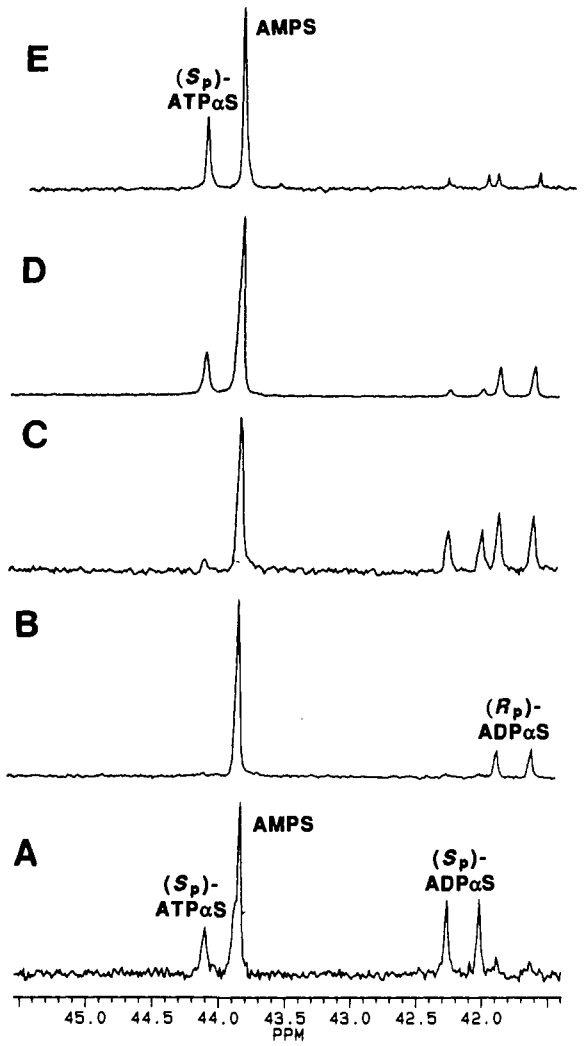

Figure 2. ^{31}P NMR analysis (at 121.5 MHz) of stereospecificity at neutral pH. Only the regions of the P_α resonances are shown. The starting reaction mixture (2 mL) consisted of 22 mM AMPS, 75 mM ATP, 45 mM $Mg(NO_3)_2$, and ca. 0.04 mg of WT or ca. 0.4 mg of R44M, in a 50 mM Tris buffer containing 50 mM KCl and 2.5 mM EDTA, pH 7.8. The temperature in the NMR probe was 30 °C (A) WT AK, at 4 h (midpoint of acquisition) after addition of the enzyme; (B) R44M, 2 h; (C) addition of ADPαS (R_p/S_p = 1:2) to B, 4 h; (D) continuation of C, 9 h; (E) continuation of D, 70 h. The free induction decay was acquired with broad-band decoupling and processed with 0.5- or 1-Hz exponential multiplication. The chemical shifts are referenced to external 85% H_3PO_4. The right half of the doublet of (S_p)-ATPαS overlaps with the singlet of AMPS. The position of (R_p)-ATPαS should be upfield from AMPS. The relative chemical shifts agree with the values reported previously[4,13] (the absolute values are sensitive to pH and buffer conditions).

Since the stereospecificity in the present case is a kinetic phenomenon, it is important to follow the time course of the reaction. Otherwise complication could arise from equilibrium effects. For example, further incubation of the sample of Figure 2D resulted in the conversion of the R_p isomer of ADPαS to the S_p isomer of ATPαS (Figure 2E), apparently via back reaction to AMPS and transient formation of (S_p)-ADPαS. Also, the stereospecificity should not be considered to be 100%. In Figure 2A, for example, a small amount of (R_p)-ADPαS (ca. 15% of total ADPαS, or ca. 8% of total products) can also be detected.

Overall, our results are significant in several aspects: (a) Site-directed mutagenesis can be used to manipulate the P-stereospecificity of enzymes and produce useful diastereomers of phosphorothioates. In the present case, R44M provides a direct way to synthesize pure (R_p)-ADPαS from AMPS, which is simpler than the previously available procedure of chemically synthesizing (R_p+S_p)-ADPαS followed by enzymatic removal of the S_p isomer.[6] (b) A reversal, or a significant perturbation in the stereospecificity, is strong evidence that the mutated residue interacts with the phosphorothioate group. The results in this work unequivocally establish that Arg-44 interacts with the phosphoryl group of AMP during the catalytic reaction. Such delineation of the functional role of an active-site residue is a step forward from direct interpretation of the static crystal structure or the kinetic data of mutant enzymes. (c) In-depth investigation of various systems can enhance our understanding of the chemical basis of enzymatic catalysis and the biological effects of phosphorothioates.

Nitrogen-15-Labeled Oligodeoxynucleotides. 3. Protonation of the Adenine N1 in the A·C and A·G Mispairs of the Duplexes $\{d[CG(^{15}N^1)AGAATTCCCG]\}_2$ and $\{d[CGGGAATTC(^{15}N^1)ACG]\}_2$

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Nitrogen NMR of specifically labeled molecules has the potential to provide unique information about structure and interactions.[1,2] The chemical shift of an sp^2 nitrogen, for example, is strongly affected by protonation. In the case of the adenine N1, protonation is known to cause an upfield shift of ~70 ppm.[2] Thus, nitrogen NMR should allow unambiguous determination of when or if a specific (labeled) adenine N1 is protonated, regardless of the size of the molecule, within the limit of τ_c, the molecular correlation time. We synthesized the labeled molecules d[CG(^{15}N^1)AGAATTCCCG] (**1**) and d[CGGGAATTC-(^{15}N^1)ACG] (**2**), using an H-phosphonated method, and monitored the ^{15}N chemical shift of each as a function of pD over a range of ~5 to ~8.[3] The ^{15}N chemical shifts were detected indirectly through the corresponding H2 atom by using a ^{1}H-detected heteronuclear 2D NMR experiment.[4] This technique reduces dramatically the NMR time required, relative to direct ^{15}N detection, to achieve a given level of sensitivity. In the ^{1}H–^{15}N 2D NMR spectrum obtained, the observed signals provide both ^{1}H and ^{15}N chemical shifts for the individual heteronuclear *J*-coupled spin systems.

The A·C[5-11] and A·G[11-20] mispairs have been studied extensively,

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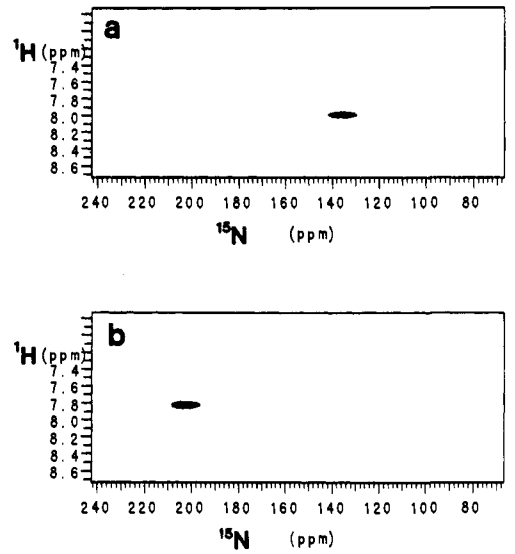

Figure 1. 2D ^{1}H–^{15}N correlation spectra of d[CG(^{15}N^1)-AGAATTCCCG] (**1**) at 31 °C in D_2O containing 0.1 M NaCl, 10 mM phosphate, 0.1 mM EDTA, at pD 5.0 (a) and at pD 7.8 (b) obtained by using an in-phase heteronuclear multiple quantum correlation experiment: 90°(H_x)–1/(4 $^2J_{NH}$)–180°(H), 180°(N)–1/(4 $^2J_{NH}$)–90°(H_x), 90°($N_{\pm x}$)–t_1/2–180°(H)–t_1/2–90°(N_x)–1/(4 $^2J_{NH}$)–180°(H), 180°-(N)–1/(4 $^2J_{NH}$)–Acq(±).[4] The spectra were recorded at a ^{1}H frequency of 500 MHz with a resolution of 7.5 ppm/point (equivalent to a t_1 acquisition time of 2.7 ms) for the ^{15}N dimension. Four transients for each FID were acquired, at a t_2 acquisition time of 128 ms, an experimental recycle time of 2.2 s, a τ delay equivalent to J-coupling of 14.5 Hz, and a total acquisition time of 10 min. To more accurately determine the ^{15}N chemical shift, higher resolution spectra were also obtained, at a similar total acquisition time, by reducing the spectral window (data not shown). The ^{15}N chemical shifts are relative to $^{15}NH_4Cl$ in 10% HCl.

including both X-ray and NMR analyses. In several instances protonation of the adenine N1 in the mispair could be inferred, but not proved. For example, X-ray data demonstrated the correct distance for H-bonding, but the proton itself is too small to be visualized.[6,9,14,15] Similarly, while ^{1}H NMR could, in principle, detect the proton of such an H-bond, in practice this has not proved to be possible, perhaps due to rapid exchange.[8,10,17] The use of nonexchangeable proton chemical shifts to establish protonation of the N1 has been proposed,[7] although some problems with this approach have been noted.[8] Nevertheless, the structures of several mismatch-containing duplexes have been well defined. In particular, the two molecules we used in this study had been extensively characterized by 2D ^{1}H NMR.[8,17]

The syntheses of **1** and **2** were carried out by an H-phosphonate method, on an 18-μmol scale, on a Biosearch 8750 synthesizer.[21] The deoxyadenosine H-phosphonate monomers were N-protected as the dibutylamidine derivatives because of the enhanced stability of the glycosidic bond afforded by this protecting group.[22,23] The [1-^{15}N]-2′-deoxyadenosine was prepared as reported previously.[24] The product molecules **1** and **2** were purified by reversed-phase HPLC both before and after detritylation and were characterized by enzymatic degradation to the constituent deoxynucleosides.[25] Details of the syntheses and purifications are available as supplementary material.

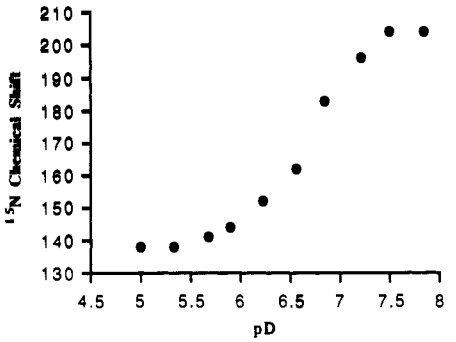

Figure 2. A plot of ^{15}N chemical shift vs pD for d[CG(^{15}N^1)-AGAATTCCCG] (**1**).

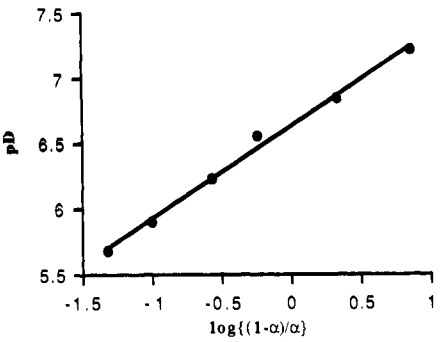

Figure 3. A plot of pD vs log [(1 − α)/α] for d[CG(^{15}N^1)-AGAATTCCCG] (**1**). The pK_D is given by the intercept of the line equation (y = 6.6363 + 0.70968x).

d[CG(^{15}N^1)AGAATTCCCG]. The ^{15}N chemical shift of **1** was monitored from pD 5.0 to 7.9.[3] Over this pD range the ^{15}N resonance is shifted upfield by 66 ppm, relative to the chemical shift at pD 7.9 (Figure 1). This is consistent with the shift expected for protonation of the adenine N1 (although in this case the "protonation" is with ^{2}H).[26] The ^{15}N chemical shift we observed for the protonation of pyridine with ^{2}H was less than 2 ppm smaller than that for ^{1}H.[27] A plot of the observed ^{15}N chemical shift vs pD, shown in Figure 2, is sigmoidal in shape and shows the apparent pK_D to be greater than 6.5. The pK_D can be determined more accurately from a plot of pD vs log [(1−α)/α] (Figure 3), which gives a straight line for which the intercept is the apparent pK_D. The quantity α is the fraction of molecules protonated at a given pD, calculated from the ratio of the chemical shift difference at that pD(relative to the high pD base line) to the overall chemical shift change, assuming flat upper and lower base lines. These data demonstrate that the apparent pK_D of the adenine N1 atom for this A·C mispair is 6.6, more than two logs higher than that for the monomer,[7,28] and establish that the low pD structure is protonated at the adenine N1. A table listing the values of ^{15}N chemical shift, pD, α, and log [(1 − α)/α] is included in the supplementary material.

d[CGGGAATTC(^{15}N^1)ACG]. The ^{15}N chemical shift of **2** was monitored from pD 4.5 to 7.0. Once again, the ^{15}N resonance was shifted upfield at low pD (supplementary material), in this case by 69 ppm. At intermediate pD values, however, the ^{15}N resonance was too broad to be detected, so that we were not able to determine the apparent pK_D of the N1 in this A·G mispair. This molecule had been shown by ^{1}H NMR to have a pH-dependent conformational equilibrium between G(anti) and G(syn) structures, at high and low pH, respectively.[17] This equilibrium may be the source of the line broadening that we observed. Never-

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(28) It should be noted that the monomer pK was determined under conditions that differ both in ionic strength and in solvent composition (H_2O vs D_2O) from those used in the present work.

theless, these ^{15}N results establish that the adenine N1 is protonated in the low-pD, G(syn) structure of this A·G mispair, while in the high pD, G(anti) structure, it is not.

Summary. We have shown that the adenine N1 atom in the A·C and A·G mispairs studied is protonated at pD values above those of the monomer p*K*. These experiments provide the first direct evidence for protonation of a specific nitrogen in a mispair and demonstrate further the potential utility of ^{15}N labeling to probe DNA structural details.

Acknowledgment. This work was supported by grants from the National Institutes of Health (GM31483) and the Busch Memorial Fund, and an American Cancer Society Faculty Research Award to R.A.J.

Supplementary Material Available: Details of the syntheses and purifications of **1** and **2**, together with ^{15}N NMR and ^{1}H NMR spectra (7 pages). Ordering information is given on any current masthead page.

[1,3] Sigmatropic Rearrangement of Allyl Vinyl Ethers at Ambient Temperature in 3.0 M Lithium Perchlorate–Diethyl Ether

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The intervention of the [1,3] sigmatropic rearrangement during the course of a Claisen rearrangement is a rare event, witnessed previously only in a few cases[1] where the typical [3,3] process is energetically and/or sterically unfavorable.[2,3] We report that allyl vinyl ethers undergo unprecedented [1,3] sigmatropic rearrangement at ambient temperature in 3.0 M lithium perchlorate–diethyl ether.[4]

In a preliminary set of experiments, a 0.2 M solution of allyl vinyl ether **1** in 3.0 M lithium perchlorate–diethyl ether was shown to undergo exclusive [1,3] sigmatropic rearrangement after 1 h at ambient temperature, giving rise to a 90% yield of **2** and **3** in a 5:1 ratio. Use of 5.0 M lithium perchlorate–diethyl ether gave rise within 10 min to **2** and **3** in the same ratio. Use of 1.5 M lithium perchlorate in diethyl ether gave rise to a 98% yield of **2** and **3** in a ratio of 7.5:1; however, under these conditions the rate of the reaction was slowed, requiring 24 h to go to completion. Interestingly, exposure of the corresponding C(12) epimeric allyl vinyl ether to 3.0 M lithium perchlorate in diethyl ether gave rise after 1 h to a 94% yield of the [1,3] rearranged products **2** and **3** in a 5:1 ratio. Our observations stand in sharp contrast to our earlier studies on the Claisen rearrangement of **1** in aqueous medium,[5] wherein unprotected allyl vinyl ether **1** in water–methanol (2.5:1) was observed to undergo exclusive [3,3] sigmatropic rearrangement at 80 °C. The facile [1,3] rearrangement observed above, coupled with the synthetic potential of this novel carbon–carbon bond forming reaction, led us to probe the generality of lithium perchlorate–diethyl ether induced [1,3] sigmatropic rearrangements of allyl vinyl ethers.

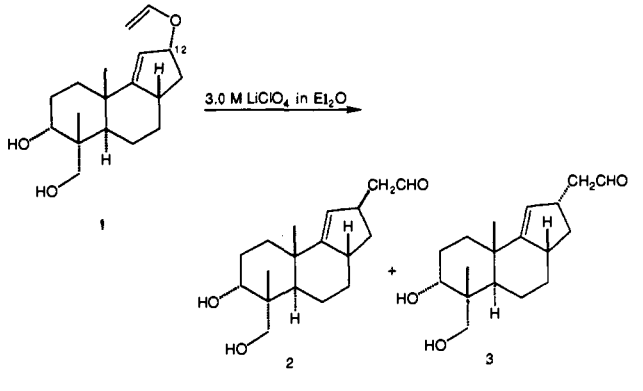
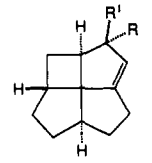
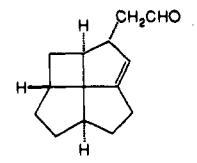
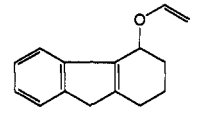
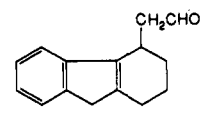
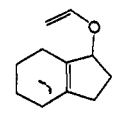
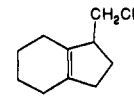
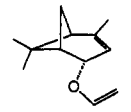
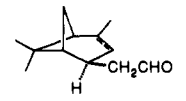
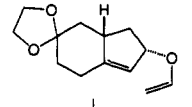
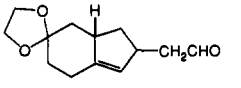
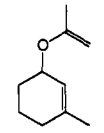
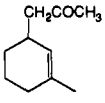


Table I. [1,3] Rearrangement of Allyl Vinyl Ethers Employing 3.0 M Lithium Perchlorate in Diethyl Ether[a]

entry	substrate	time, h	product(s)	% yield[b]
1	 R=H, R¹= ·OCH=CH₂	1		90[c]
2	R= ·OCH=CH₂, R¹=H	1		72[d]
3		1		70[e]
4		1		94
5		24		94
6		1		83[f]
7		1		86

[a] All reactions were conducted at ambient temperature employing a 0.2 M solution of allyl vinyl ether in 3.0 M lithium perchlorate–diethyl ether. [b] Isolated yields. [c] Approximately 3% of the [3,3] sigmatropic product was isolated. [d] Approximately 2% of the [3,3] sigmatropic product was obtained. [e] Approximately 1% of the Claisen product along with 28% of an elimination product was observed. [f] The β:α ratio was 5:1.

The procedure employing 3.0 M lithium perchlorate in diethyl ether to promote [1,3] rearrangement of allyl vinyl ethers is applicable to a variety of substrates (Table I). In all cases studied, [1,3] sigmatropic rearrangement is the major reaction pathway; however, traces of products arising from [3,3] sigmatropic rearrangement and, in a few cases, elimination with formation of dienes have been observed.

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(2) For examples of Pd(0)-catalyzed [1,3] rearrangements of 5-vinyl-2-alkylidenetetrahydrofurans, see: Trost, B. M.; Runge, T. A. *J. Am. Chem. Soc.* **1981**, *103*, 7559.

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