Curvature of Dinucleotide Poised for Formation of Trinucleotide in Transcription with *Escherichia coli* RNA Polymerase

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ABSTRACT: A frequently used schematic model of transcriptional elongation shows an RNA polymerase molecule moving along a linear DNA. This model is of course highly idealized and not compatible with promoter sequences [Gralla, J. D. (1991) *Cell 66*, 415−418; Schleif, R. (1992) *Annu. Rev. Biochem. 61*, 199−223] and regulatory proteins [Koleske, A. J., and Young, R. A. (1995) *Trends Biochem. Sci. 20*, 113−116; Dunaway, M., and Dröge, P. (1989) *Nature 341*, 657−659; Müller, H. P., Sogo, J. M., and Schaffner, W. (1989) *Cell 58*, 767−777] located some distance away from the point of transcription initiation [Karsten, R., von Hippel, P. H., and Langowski, J. (1995) *Trends Biochem. Sci. 20*, 500−506]. These circumstances lead to the expectation of curvature along the DNA strand and require looping between sometimes distant points. We have now shown curvature in a dinucleotide formed at the very onset of transcription when it is poised for reaction with a mononucleotide to form a trinucleotide. The curvature became evident from the demonstration that a metal ion bound with a mononucleotide in the *i*+1 (elongation) site is approximately equidistant from bases at the 5′ end (*i*−1 site) and 3′ end (*i* site) of the dinucleotide. Similar results were obtained with three different dinucleotides and four mononucleotides. Curvature of the RNA initiate may reflect curvature of the DNA to which it is bound. These studies show curvature to be a significant feature in the interaction between DNA template and RNA elongate even at the very beginning of transcription.

In the process of transcribing DNA, RNA polymerase must initially bring together two nucleotide (NTP) substrate molecules for internucleotide bond formation and subsequently add further NTP substrates to the 3′ terminus of the evolving RNA chain as it progresses along the template. We used the *i* site nomenclature first introduced by Yager and von Hippel (*7*) as an ideal for identifying NTPs during the transcription process. In this labeling system, the 3′ terminus of the RNA chain is located in what is described as the *i* subsite of the enzyme's active site. The incoming or next NTP to be incorporated is located in the *i*+1 subsite, and both of these subsites are characterized by the presence of a divalent metal: Zn(II) in *i* and Mg(II) in *i*+1. Previously transcribed bases can therefore be described as *i*−1 to *i*−*n* for a transcript of *n* nucleosides. For the stage to be investigated in this paper, the addition of a third nucleotide to an existing dinucleoside monophosphate, or dinucleotide (NpN), the nomenclature is illustrated for UTP added to UpA, as follows:

U p A UTP

i-1 i i+1

We have previously studied the very first step in transcription (*8*), the formation of an NpN from NTPs in both *i* and *i*+1 sites. In this earlier work, we asked whether the failure of Watson−Crick base pairing alone to account for the fidelity of transcription is compensated by an action of the enzyme itself, and found that indeed the enzyme undergoes conformational change in its active site to enhance fidelity (*8*). We used Mn(II) as a probe of geometric relationships among the substrates of *Escherichia coli* RNA polymerase, by replacing the Zn(II) in the *i* site and/or the Mg(II) in the *i*+1 site with this metal, whose paramagnetism can be used for distance determination by EPR and NMR methods.

In this study of systems designed for the formation of trinucleotides, we ask primarily the following question. Is there a linear relationship between the three nucleotides poised for trimer formation (i.e., two nucleotides with the bond already present and the third NTP about to enter into bond formation)? Or is there a curvature?

To answer this question, we placed three different dinucleotides into the *i* site in various experiments: UpA, ApU, and ApA. These NpNs in the *i* site actually contain the 5′ and 3′ bases in *i*−1 and *i*, respectively. We placed a variety of mononucleotides into the *i*+1 site: UTP, GTP, CTP, and ATP, as well as α,*β*-methylene-ATP (the latter having the oxygen between the α and *β* phosphorus replaced by CH_2). We then placed Mn(II) into the *i*+1 site and determined distances from that metal ion to protons on the *i*−1 and *i* nucleotides in the dinucleotide. In a previous study, a measurement had been made with ApU, in the absence of a nucleotide in the *i*+1 site (*9*).

The use of different nucleotides in the *i*+1 site enables us to ask a second question. Does the geometry of the dinucleotide *i*-*i*−1 depend on the nature of the *i*+1 NTP, including its complementarity or noncomplementarity to the DNA base to be copied? [We know that the *i*+1 site itself

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Table 1: Distance of *i* Subsite UpA Protons to *i*+1 Subsite Mn (Å)

i+1 subsite substrate	U-H_5	U-H_6	U-$H_{1'}$	A-H_2	A-H_8	A-$H_{1'}$
GTP[b]	12 ± 2	12 ± 2	12 ± 2	9.7 ± 0.4	8.9 ± 0.6	12 ± 0.9
CTP	12[d]	13[d]	11[d]	9.9[d]	8.9[d]	12[d]
ATP	*c*	14[d]	13[d]	9.3[d]	8.6[d]	13[d]
α,β-M-ATP[a,b]	11 ± 2	13 ± 2	12 ± 3	9.1 ± 0.4	8.6 ± 0.5	11 ± 0.9

[a] α,β-M-ATP is α,β-methylene-ATP. [b] Data are the average of three experiments. [c] Data not obtained. [d] Data from only one experiment available.

Table 2: Distance of *i* Subsite ApU Protons to *i*+1 Subsite Mn (Å)

i+1 subsite substrate	A-H_2	A-H_8	A-$H_{1'}$	U-H_5	U-H_6	U-$H_{1'}$
GTP[b]	9.7 ± 0.2	9.0 ± 0.3	11.3 ± 0.9	13 ± 1	16 ± 2	14 ± 1
CTP[b]	9.9 ± 0.3	9.3 ± 0.3	13 ± 1	14 ± 2	14[d]	15 ± 1
UTP[b]	10.1 ± 0.3	9.4 ± 0.2	13 ± 2	14 ± 1	*c*	16[d]
α,β-M-ATP[a,b]	9.1 ± 0.2	8.6 ± 0.2	11.8 ± 0.4	11.9 ± 0.3	14.5 ± 0.4	12.5 ± 0.4

[a] α,β-M-ATP is α,β-methylene-ATP. [b] Data are the average of two experiments. [c] Data not obtained. [d] Data from only one experiment available.

Table 3: Distance of *i* Subsite ApA Protons to *i*+1 Subsite Mn (Å)

i+1 subsite substrate	5′ A-H_2	5′ A-H_8	5′ A-$H_{1'}$	3′ A-H_2	3′ A-H_8	3′ A-$H_{1'}$
GTP[b]	9.0 ± 0.2	8.3 ± 0.2	12 ± 0.3	9.1 ± 0.3	8.1 ± 0.2	12 ± 1
UTP[b]	9.6 ± 0.3	9.0 ± 0.2	12[d]	10.0 ± 0.7	8.8 ± 0.2	*c*
α,β-M-ATP[a,b]	8.2 ± 0.3	7.9 ± 0.2	11 ± 1	8.4 ± 0.3	7.6 ± 0.2	10 ± 1

[a] α,β-M-ATP is α,β-methylene-ATP. [b] Data are the average of two experiments. [c] Data not obtained. [d] Data from only one experiment available.

is affected by complementarity (*8*); is the other site affected as well?] If this second question is answered in the negative, and the dinucleotide geometry is the same in the presence of different *i*+1 mononucleotides, the use of the various NTPs will simply constitute a series of checks on that geometry.

MATERIALS AND METHODS

The methodology consisted of the isolation and purification of *E. coli* RNA polymerase as previously described (*10*). Activity was assayed by the method of Chamberlin et al. (*11*), which measures the percentage of active, DNA-bound enzyme, and ranged from 50 to 80%, as previously indicated (*10*). NMR samples were prepared in 50 mM Tris/200 mM KCl buffer, with D_2O substituted for water solvent. The enzyme was deuterated by a repeated cycle of dilution with D_2O buffer and concentration by vacuum dialysis. Two DNA templates were employed. Poly(dAdT)·poly(dAdT) was first dialyzed against H_2O buffer to remove a recurrent Mn binding impurity, concentrated by vacuum dialysis against the same buffer, and then evaporated to dryness. The residue was redissolved in a volume of D_2O equal to that of the solution prior to evaporation. Poly(dA)·poly(dT) was first sonicated to reduce its mean chain length to the order of several hundred base pairs to prevent a gelling phenomenon encountered in NMR experiment solutions, which can be attributed to the high concentrations employed. Following sonication, the solution was treated with s_1 nuclease to remove any single-stranded material. Trace metal impurities were removed from all solutions by treatment with Chelex 100. The concentrations were 1 mM NTPs, 20 μM RNA polymerase, 3 mM NpN, 1 mM DNA template, and 0−20 μM Mn. The concentration of template employed was equivalent to 50 base pairs per enzyme molecule. Longitudinal relaxation times (T_1) of the proton nuclei of ApA, ApU, and UpA were measured as a function of Mn concentration at 200 MHz on a Varian XL-200 NMR spectrometer by using a $180°-\tau-90°$ inversion recovery pulse sequence (*12*). To obtain the highest possible precision in measurements presented in Tables 1−3 (between ±2 and ±3%), we used the same enzyme preparation and the same reagent solutions (except, of course, the mononucleotide solution) in experiments with a given dinucleotide. Precision is more important than accuracy in these experiments designed for a comparison of distances.

For the NMR measurements to be feasible, conditions that permit any reaction to take place in the solution must be avoided; otherwise, the original components of the system will disappear while various products of RNA synthesis will be found. When noncomplementary NTPs are present, reaction is precluded. We studied complementary cases with αβ-methylene-ATP instead of ATP, thus again preventing a reaction. In a preliminary experiment, we did use ATP with ApU, so that reaction could take place. In this instance, the ApU peaks were observed prior to the addition of Mn, but these disappeared upon the addition of Mn. Thus, the enzyme was active under the conditions of the NMR experiments, at the Mn concentrations used, when the reagents made such activity possible.

The theoretical basis for the measurement of distances on the active site of RNA polymerase was described extensively by Bean et al. (*9*) and also in a previous paper from this laboratory (*13*). The current study differs in that we observe by NMR only the NpN in the *i* subsite. The complex of interest is that comprised of enzyme template, NpN, NTP, and Mn(II). Though this complex is in equilibrium with its components, dissociation equilibrium constants (*14*, *15*) show that most of the enzyme is present in this complex. In a previous paper (*13*), it was demonstrated through water relaxation rates that the presence of template does not significantly affect the affinity of the metal ion toward the enzyme. Neither NTP nor NpN binds Mn(II) sufficiently strongly (relative to the enzyme) to warrant steps to correct for binary Mn(II) NTP (*14*) and Mn(II) NpN (*9*) complexes. The effect of

unbound or nonspecifically bound Mn would be unlikely to be observed as a constant perturbation of substrate protons, and would therefore not lead to constant distances to the NpN.

Molecular modeling for Figure 1 was performed on a Silicon Graphics/Indigo XS 24 workstation using the CHARMm version 22 force feed (*16*) with the Quanta release 3.3.1 molecular simulation package. Our experimentally measured distance constraints were introduced into the CHARMm energy term as harmonic potentials. The charge used for the NTPs was that at pH 8, at which the NMR studies were undertaken. Low-energy conformations for modeled systems were obtained by minimization using the Adopted Basis Newton−Raphson (ABNR) method (*16*).

The structure shown in Figure 1 represents the best compromise between the measured distances obtained in this paper and the maintenance of the relatively invariant aspects of the molecule, such as the planarity, bond angles, and bond lengths of the heterocycles. In setting up the distance constraints, the distances with smaller standard deviations were more strongly enforced than those with larger standard deviations.

RESULTS

We first consider the results (using Table 1) obtained for the distances from the *i*+1 Mn to UpA (*i*−1 U and *i* A). We used the poly(dAdT)·poly(dAdT) template, and in the *i*+1 site GTP, CTP, ATP, and α,*β*-methylene-ATP. Since the U and A in UpA are aligned with A and T sites on the DNA, the *i*+1 site has an A in DNA lined up with G, C, or A, none of which is Watson−Crick complementary to the DNA-A.

The distances from the Mn to H_5 and H_6 in *i*−1 U range from 11 to 14 Å. These are consistently much longer distances than those from the Mn to H_2 and H_8 of *i* A; these range from 8.6 to 9.9 Å. If UpA were the only dinucleotide studied, we could erroneously be tempted to conclude that the *i*−1 base is further removed than the *i* base from the *i*+1 Mn, consistent with a linear array of bases from *i*−1 to *i* to *i*+1. However, the distances to $H_{1'}$ in both U and A are quite similar to each other, varying from 11 to 13 Å, and these results do not support a linear array.

The base in the *i*+1 site does not appear to have a significant effect on the distances to the dinucleotide. Also, the replacement of normal ATP with one that has the oxygen between the α and *β* phosphorus atoms replaced by CH_2 has no effect on the distances.

We next consider distances from the *i*+1 Mn to ApU (A in *i*−1 and U in *i*, the opposite of UpA), again with poly(dAdT)·poly(dAdT) as the template, and in the *i*+1 site, we used GTP, CTP, UTP, and α,*β*-methylene-ATP (Table 2). Here the A and U in ApU bind to T and A in DNA, respectively, so that the next DNA base, a T, would require an A for complementarity. We used the ATP analogue instead of native ATP for the complementary case, to prevent a transcription reaction that would complicate our NMR results, and the results in Table 1 show that native ATP and its analogue give similar results. The other mononucleotides are noncomplementary and could be used without modification.

In Table 2, as in Table 1, the H_5 and H_6 protons on U are again further away than the H_2 and H_8 protons on A from the *i*+1 Mn (Table 2). In fact, the Mn−U and Mn−A distances are quite similar in the two systems. However, since the positions of the bases are reversed on the NpN (ApU in Table 2 vs UpA in Table 1), the observed *i*−1 protons in ApU are actually closer than the *i* protons to *i*+1 Mn. Clearly, these results are not consistent with linearity. The nature of the base and the protons measured thereon, not its position in the NpN, appear to determine this distance; clearly, a curvature of the ApU is indicated! The nature of the mononucleotide in *i*+1 again has little effect on the distances to the bases in the dinucleotide, except that the presence of the complementary ATP analogue leads to slightly shorter distances than those observed with the noncomplementary mononucleotides.

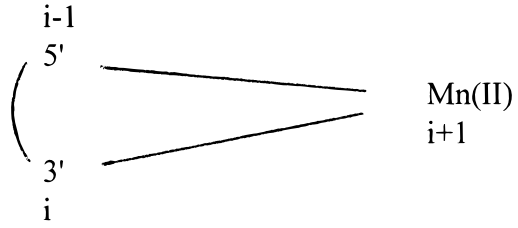
Distances from *i*+1 Mn to *i* ApU, with the *i*+1 site empty of nucleotide, had been previously determined. Distances to the adenosine were similar to those found here, but distances to the uridine were similar to those to adenosine, and therefore different from those found here (*9*). Thus, some sort of curvature seems to occur also in the absence of an *i*+1 nucleotide, but the geometry of the curvature is changed when a mononucleotide is placed into the *i*+1 site.

Experiments to this point have demonstrated that H_5 and H_6 on U are always further from the *i*+1 Mn than H_2 and H_8 on A, whatever the placement of the two bases in either the 5′ or 3′ position on the dinucleotide. We now investigate ApA, where the two bases are identical; to be consistent with the UpA and ApU results, the distances to the two A bases should be the same. In this case, we selected poly(dA)·poly(dT) as the template so that bases on ApA could bind to the T strand of the template; a T would then also be available for binding in the *i*+1 site. In the *i*+1 site, UTP and GTP contain noncomplementary bases while α,*β*-methylene-ATP bears a complementary base.

The results in Table 3 demonstrate that the distances from the *i*+1 Mn to the H_2 and H_8 protons are about the same for the 5′ and 3′ positions, and indeed are also the same as the distance of the Mn from the A base in the 3′ position of UpA and the 5′ position of ApU, i.e., in the 8−10 Å range. Again, the nature of the *i*+1 mononucleotide does not appear to influence the distances, at least in the noncomplementary cases (GTP and UTP).

DISCUSSION

Curvature at the Onset of Transcription. We have thus obtained evidence that in a very early stage of transcription, in which a dinucleotide is in the *i*-*i*−1 site and a mononucleotide in *i*+1 is about to be added to the forming RNA chain, the dinucleotide is not in a linear relationship with respect to the incoming NTP. The evidence consists of the demonstration that the 5′ and 3′ bases of several dinucleotides are essentially equidistant from the metal bound to the incoming mononucleotide in the *i*+1 site. This relationship can be visualized schematically as follows:



Linearity would require the 5′ base to be further than the 3′ base from the metal. The structure of one of the dinucleotides, ApU, is shown in stereo in Figure 1.

Evidently, the dinucleotide forms a curvature during the transcription process, and the portion of the DNA to which it is bound is probably also curved. Since various transcriptional phenomena require such curvature (*6*), this result need not be surprising, the linear schematic notwithstanding.

The equidistance of the *i*+1 Mn from the 5′ and 3′ ends of the dinucleotide is confirmed by studies with three dinucleotides, each of which has been associated with several mononucleotides in the *i*+1 site. The results are essentially the same with all dimers and mononucleotides, thus confirming the conclusion of curvature under all of these conditions. Thus, in Table 1, distances to the A-H_2 vary from 9.1 to 9.9 Å and to A-H_8 from 8.6 to 8.9 Å. In Table 2, distances to A-H_2 vary from 9.1 to 10.1 Å and to A-H_8 from 8.6 to 9.4 Å. In Table 3, distances to A-H_2 vary from 8.2 to 10.0 Å and to A-H_8 from 7.6 to 9.0 Å. Distances to U-H_5 and U-H_6 are all above 11 Å and usually between 12 and 14 Å in both 3′ and 5′ positions.

Distance from a Metal to a Nucleoside Base Depends on the Base, Not on Its Position on the NpN. We attribute no particular significance to the fact that the distances from the metal ion to U-H_5 and U-H_6 are greater than those to A-H_2 and A-H_8. The bases have a very different structure, and the nuclei detected by the NMR in uridine are at different positions on the base than those detected in adenosine. It should be noted in this regard that the distances from the metal to the $H_{1'}$ in adenosine and uridine are similar (and not unlike those to the uracyl protons) for virtually all combinations of *i* dinucleotide and *i*+1 mononucleotides. The differences in the distances to the observed nuclei in the two bases can thus be attributed to the distances to those nuclei rather than to the bases as a whole. The fact that the observed distances to uridine H_5 and H_6 and to adenosine H_2 and H_8 are different from each other, but remain the same whether on the 3′ or 5′ side of the dinucleotide, increases our confidence that curvature occurs in the dinucleotide on the enzyme site.

Are We Observing Enzyme That Is Bound to Template? This question is related to another question, whether the RNA is base paired with the DNA. If base pairing occurs, the enzyme is of course bound to the template. We believe that our previous studies on "a structural model for fidelity in transcription" (*8*) have rather unequivocally demonstrated such base pairing under our experimental conditions, since we showed that the enzyme assumes one conformation for complementary structures and a different conformation for noncomplementary ones. This would not be possible in the absence of base pairing. In the current study, we have been able to measure constant distances from Mn in the *i*+l site to NpN in the *i* site (based on constant perturbation of the NpN protons by the Mn). Metal ions nonspecifically bound to enzyme in the absence of template would be unlikely to lead to constant distances to the NpN.

Comparison of Structures of Unbound Dinucleotides with Those Bound to Enzyme. To better understand the significance of the dinucleotide curvature when enzyme-bound, it is of some interest to compare it with the structure of unbound dinucleotides. It has been shown that the dinucleotides assume a variety of structures that have an important common feature: they are designed to make hydrogen bonding possible between bases.

Let us first consider UpA. Two independent crystal structures of this molecule (*17*−*19*) have led to the same result: the crystals contain two different structures in which the adenosines in the two conformers are self-paired in a Hoogsteen arrangement. The crystal structure of ApU, on the other hand (*20*), contains one conformer that produces a double helix through hydrogen bonding of the A of each ApU to a U in another ApU, and of each U to an A. In both of these molecules, there is greater or lesser curvature to place the base in the most favorable position for some type of hydrogen bonding. NMR studies of ApA (*21*) have shown that the molecules are arranged in such a manner that base stacking between the adenosines is generated.

There is thus considerable flexibility in the primary structure of these dinucleotides to enable them to bond in whatever manner is required to produce a stable intermolecular arrangement; this phenomenon certainly comes as no surprise.

It should therefore be no surprise that the flexibility of the dinucleotides should make it possible for them to assume a variety of configurations when bound to other molecules, such as the RNA polymerase enzyme and DNA template. No intramolecular restraints prevent these dinucleotides from arranging themselves in more or less linear or more or less curved structures, as the situation may require. We have seen that the dinucleotides do assume a curvature when bound to the enzyme. Such curvature occurs both in the absence (*9*) and in the presence (this paper) of mononucleotide in the *i*+1 site, but the presence of the *i*+1 nucleotide leads to a differently curved structure compared to that in its absence.

The results indicate that all of the components of the enzyme system, enzyme itself, template, dinucleotide in the *i* site, and mononucleotide in the *i*+1 site, are necessary for the dinucleotide to attain the specific curvature that is optimal for elongation at the active site of the enzyme.

It may be worth pointing out that dinucleotides do not necessarily form curved structures when bound to substances other than this enzyme. For example, ApA forms a highly extended conformation when bound to proflavine (*22*). The adenosines from adjacent dinucleotides again form Hoogsteen base pairs, and each of these base pairs is separated by two proflavines, with adenosine pairs and proflavines together producing an extensive stacked structure. This comparison demonstrates that there is no intrinsic tendency of a dinucleotide to become either curved or extended in combination with other molecules, and the curvature in the RNA polymerase site thus occurs as a result of a specific structural requirement at that site.

If one speculates about the nature of this structural requirement, the DNA template seems to be of obvious interest, since the initiation process of RNA synthesis involves binding on a much-discussed "open-promoter" or "bubble" on the template (*23*, *24*). It is easy to visualize the curvature in the dinucleotide as reflecting a similar curvature or bend on the DNA template. In fact, the usual schematic of the DNA bubble does place such a bend at the point at which RNA synthesis begins, although it would not be essential in this scheme that curvature must exist between the first and second nucleotide of the nascent RNA. The results described here indicate that it does exist there, if we

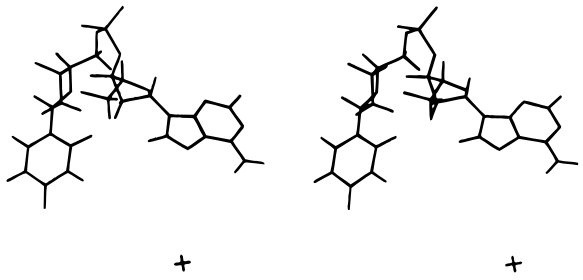


FIGURE 1: Stereo representation of ApU in the presence of *E. coli* RNA polymerase, template, and substrates as indicated in the text and Table 2. Uracil is on the left and adenine on the right; the two ribose components and phosphate are in the center. Mn is represented by the cross at the bottom.

can assume that the RNA curvature is responsive to one in DNA. We emphasize, however, that our actual data are for the RNA alone.

There have been many recent studies of DNA bending at promoter sites [such as the σ^{54} (*25*)], but these bends generally occur some distance away from the RNA polymerase active site. The curvature studied here is reminiscent of these bends, but, so far as we know, there is no other definitive evidence for the curvature at the site we have investigated.

Structural Model Shown in Figure 1. The distances obtained in this study lead to the structure shown in Figure 1 for ApU. It should be noted that this structure is a close approximation, but an approximation nevertheless, considering the variability of the results in Table 2, upon which Figure 1 is based. This variability may be due in part to the presence of different NTPs in the *i*+1 site, but is due also in part to the experimental error indicated by the standard deviations shown in Table 2. Thus, although Figure 1 represents a reasonably accurate picture of the structure of the dinucleotide in the enzyme−template−substrate complex, it should not be considered more precise than the data from which it is obtained, and we have therefore not attempted to use the variability of the data to determine the degree of flexibility of the molecule within the enzyme active site.

Distances within the i+1 Site Differ for Complementary and Noncomplementary Bases (8); Does Complementarity Also Affect the Distances from i+1 Mn to i NpN? In most of these studies, the *i*+1 mononucleotide is noncomplementary to the DNA base, but the ATP analogue in Tables 2 and 3 is complementary, and in all of those instances the distances are slightly shorter than those for the complementary nucleotides. We do not believe that the lower number can be attributed to the replacement of O by CH_2, since the native ATP and the ATP analogue give similar results with UpA, when they are both noncomplementary (in Table 1); we interpret this result as indicating that the ATP analogue binds to the enzyme in about the same way as native ATP. The slightly smaller distances, in the complementary cases, from the *i*+1 metal to the *i* substrate, are consistent with the requirement of facilitating bond formation only for the complementary nucleotides, in line with our previous study (*8*). We note these slightly shorter distances because of their consistency, but do not draw any definite conclusion from them because the differences are so small. At any rate, the comparisons between the distances from the *i*+1 Mn to 5′ and 3′ ends of the dinucleotide are valid for complementary as well as noncomplementary forms, and contribute to the validation of the curvature of the dinucleotide.

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SUPPORTING INFORMATION AVAILABLE

Coordinates of the structural model shown in Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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