The [4Fe-4S] Cluster Domain of the Nitrogenase Iron Protein Facilitates Conformational Changes Required for the Cooperative Binding of Two Nucleotides†

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ABSTRACT: MgATP binding and hydrolysis are central to all reduction reactions catalyzed by nitrogenase. The iron (Fe) protein component of nitrogenase is a homodimeric protein with a bridging [4Fe-4S] cluster and two nucleotide binding sites, one on each subunit. This work presents evidence that the [4Fe-4S] cluster domain of the nitrogenase Fe protein functions as a hinge region between the two nucleotide binding domains, participating in the cooperative binding of two nucleotides. Alanine residues at position 98 (located near the [4Fe-4S] cluster) of the *Azotobacter vinelandii* Fe protein were changed by means of site-directed mutagenesis to Val (V) and Gly (G), and the resulting altered proteins were purified and characterized. While the wild-type and A98G Fe proteins were found to bind two nucleotides (MgATP or MgADP) with strong cooperativity (Hill coefficient of 2), the A98V Fe protein was found to bind one nucleotide with no apparent cooperativity. The binding of two nucleotides to the wild-type Fe protein is known to induce protein conformational changes which are reflected as changes in the properties of the [4Fe-4S] cluster, including a change in the redox potential of the [4Fe-4S] cluster of −120 mV for MgATP binding (−300 to −420 mV) and of −160 mV for MgADP binding (−300 to −460 mV). The binding of one nucleotide to the A98V Fe protein was found to result in only half the lowering of the redox potential, with MgATP binding resulting in a −80 mV change (−280 to −360 mV) and MgADP binding resulting in a −50 mV change (−280 to −330 mV). Results from ^{1}H NMR, EPR, and CD spectra, along with Fe chelation rates, were all consistent with the binding of a single nucleotide to the A98V Fe protein inducing a partial conformational change. Finally, the A98V Fe protein with one nucleotide bound, still bound to the molybdenum−iron protein but did not support MgATP hydrolysis, electron transfer, or substrate reduction. A model is discussed in which the [4Fe-4S] cluster domain can be viewed as a hinge region between the two nucleotide binding domains which facilitates conformational rearrangements required for the cooperative binding of a second nucleotide.

Nitrogenase is a two-component metalloenzyme that catalyzes the reduction of dinitrogen and other substrates in a reaction absolutely requiring the hydrolysis of MgATP. MgATP serves at least two critical functions in this reaction (Howard & Rees, 1994). First, the binding of two MgATP molecules to the nitrogenase iron (Fe) protein component induces protein conformational changes which ready this protein for docking and electron transfer to the molybdenum−iron (MoFe)[1] protein component (Mortenson et al., 1993). Second, the hydrolysis of a minimum of two MgATP molecules to two MgADP molecules by the Fe protein−MoFe protein complex is coupled to the transfer of a single electron from the Fe protein [4Fe-4S] cluster to the MoFe protein, first to the [8Fe-7S] cluster and then to the active site iron−molybdenum cofactor (FeMoco) for reduction of substrates (Lowe et al., 1993; Peters et al., 1995). The hydrolysis of MgATP in the nitrogenase complex, in an unknown mechanism, accelerates intercomponent electron transfer and the subsequent dissociation of the nitrogenase components (Lanzilotta et al., 1996). Upon dissociation from the MoFe protein, the oxidized Fe protein is reduced and the two MgADP molecules are exchanged for two MgATP molecules, readying the Fe protein for another round of docking, MgATP hydrolysis, and electron transfer (Hageman & Burris, 1978).

The Fe protein is a homodimeric protein with the two identical subunits bridged at one end by the [4Fe-4S] cluster (Georgiadis et al., 1992). This surface of the Fe protein appears to comprise most of the docking interface with the MoFe protein (Lowery et al., 1989; Willing & Howard, 1990; Wolle et al., 1992b; Seefeldt, 1994). The two nucleotide binding sites on the Fe protein occur on each subunit, approximately 19 Å away from the [4Fe-4S] cluster and MoFe protein docking interface (Georgiadis et al., 1992). Understanding how the MgATP binding and hydrolysis events are communicated to the [4Fe-4S] cluster and MoFe protein docking interface to stimulate electron transfer and component protein dissociation is essential to defining a detailed mechanism for these complex reactions. It is known that the binding of two nucleotides to the Fe protein induces

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[1] Abbreviations: Fe protein, iron protein of nitrogenase; MoFe protein, molybdenum−iron protein of nitrogenase; EPR, electron paramagnetic resonance; CD, circular dichroism; ^{1}H NMR, proton nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BPS, bathophenanthrolinedisulfonic acid; IDS, indigodisulfonate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; E_m, midpoint potential.

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protein conformational changes which influence the properties of the [4Fe-4S] cluster some 19 Å away (Mortenson et al., 1993). Such changes in the properties of the [4Fe-4S] cluster have been monitored by EPR (Zumft et al., 1973; Lindahl et al., 1987), circular dichroism (CD) (Stephens et al., 1979; Ryle et al., 1996a), ^{1}H NMR (Meyer et al., 1988; Lanzilotta et al., 1995a), Fe chelation (Walker & Mortenson, 1974; Ljones & Burris, 1978), and shifts in midpoint potential (Zumft et al., 1974; Watt et al., 1986). The protein chain within the Fe protein from Asp 125 (at the nucleotide binding site) to Cys 132 (a ligand to the [4Fe-4S] cluster) has recently been identified as one pathway for communicating nucleotide binding to the [4Fe-4S] cluster and to the MoFe protein docking interface (Ryle & Seefeldt, 1996). An interesting feature of the binding of two nucleotides to the Fe protein is the observation of strong positive cooperativity. Hence, the binding of the first nucleotide greatly increases the affinity for the binding of the second nucleotide. This strong cooperativity in binding two nucleotides has been observed in direct binding studies (Cordewener et al., 1985; Ryle et al., 1995; Lanzilotta et al., 1995b), substrate reduction rates (Hageman & Burris, 1978), and cluster chelation rates (Ljones & Burris, 1978). These observations suggest that the binding of a nucleotide to one subunit of the Fe protein is communicated to the other subunit nucleotide binding site. An examination of the X-ray crystal structure of the *Azotobacter vinelandii* Fe protein (Georgiadis et al., 1992) indicates that direct interactions between the two nucleotide binding sites seem unlikely. Rather, it would appear that the communication between the two nucleotide binding sites could occur through the covalently bound [4Fe-4S] cluster. Such a model would be consistent with earlier suggestions that the [4Fe-4S] cluster region could serve as a hinge between the two subunits, functioning in protein conformational changes (Georgiadis et al., 1992; Howard & Rees, 1994).

In the present work, we have probed the possible function of the [4Fe-4S] cluster domain of the Fe protein as a molecular hinge required for the cooperative binding of two nucleotides. A strictly conserved Ala residue at position 98 of the *A. vinelandii* Fe protein is one of only three amino acid side chains located within van der Waals contact with the [4Fe-4S] cluster (Georgiadis et al., 1992). It seemed reasonable that movement around the [4Fe-4S] cluster would be dependent upon steric constraints imposed by this residue. To explore this possibility, we have changed by site-directed mutagenesis the side chains of the two Ala 98 residues, one on each subunit, to the amino acids Gly or Val. The single proton side chain of Gly would be expected to provide less steric constraints, while the isopropyl side chain of Val would be expected to provide more steric constraints than the methyl side chain of Ala. Replacing Ala 98 with Val was found to result in an Fe protein that could bind one nucleotide, with the loss of cooperativity. In addition, it was found that the binding of a single nucleotide to the Ala 98 Val altered Fe protein resulted in approximately half of the normal changes observed in the physicochemical properties of the [4Fe-4S] cluster. These results suggest that the [4Fe-4S] cluster domain of the Fe protein functions as a hinge between the two nucleotide binding domains and that the conformational changes induced upon the binding of two nucleotides are additive.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Expression, and Purification of Fe Proteins. Site-directed mutagenesis of the gene that encodes the Fe protein subunits of *A. vinelandii* nitrogenase, *nifH*, was carried out as previously described (Jacobson et al., 1989; Seefeldt & Mortenson, 1993). Expression and purification of wild-type and altered Fe proteins were performed as previously described (Seefeldt & Mortenson, 1993). All Fe proteins were purified in 50 mM Tris buffer, pH 8.0, in the presence of 2 mM sodium dithionite. The buffers used to purify the altered Fe proteins also included 20% glycerol as a stabilizing agent (Ryle & Seefeldt, 1996). Prior to freezing, glycerol concentrations were reduced to less than 5% by dilution with buffer without glycerol. Protein concentrations were determined by a modified biuret method using bovine serum albumin as the standard (Chromy et al., 1974). All proteins were purified to homogeneity as determined from SDS gels stained with Coomassie blue (Hathaway et al., 1979). Proteins were protected from oxygen by manipulation in sealed serum vials with 2 mM dithionite or in an argon atmosphere glove box (Vacuum Atmospheres, Hawthorne, CA) with less than 1 ppm of oxygen.

Activity Assays and MgATP Hydrolysis. The rates of MgATP hydrolysis, acetylene reduction, and proton reduction were determined as previously described (Ryle & Seefeldt, 1996).

Nucleotide Binding to Wild-Type and Ala 98 Altered Fe Proteins. The affinities for nucleotide binding to reduced Fe protein samples were determined as previously described using an equilibrium column binding technique (Ryle et al., 1995) in which Fe proteins were passed through a Sephadex G-25 column (0.7 × 15 cm) equilibrated with 50 mM Tris buffer, pH 8.0, and known concentrations of nucleotides ranging from 0 to 1750 μM for MgATP or from 0 to 1100 μM for MgADP. The protein-containing fraction which eluted from the column was split into two aliquots. One aliquot was analyzed for protein concentration using the modified biuret method (Chromy et al., 1974), and the other aliquot was analyzed for nucleotide concentration by the HPLC method previously described (Seefeldt, 1994). Apparent dissociation constants (K_d) were determined by fitting the data to the Hill equation as previously described (Ryle et al., 1995).

MgATP-Dependent Chelation of Fe^{2+} from Wild-Type and Ala 98 Altered Fe Proteins. The MgATP-dependent rates of Fe^{2+} chelation from wild-type and Ala 98 altered Fe proteins were followed continuously by the formation of the Fe^{2+}−(4,7-diphenyl-1,10-bathophenanthrolinedisulfonic acid) (BPS) complex, which was monitored spectrophotometrically at 534 nm using an absorption coefficient of 22 140 $M^{-1}\cdot cm^{-1}$ (Ljones & Burris, 1978). All spectrophotometric measurements were performed on a Hewlett Packard 8452A diode array spectrophotometer.

^{1}H NMR Spectra of the Isotropically Shifted Protons for Wild-Type and Ala 98 Altered Fe Proteins. ^{1}H NMR spectra were recorded as previously described (Lanzilotta et al., 1995a). All spectra were recorded at 305 K in glass NMR tubes fitted with degassed Teflon stoppers (Wilmad, Buena, NJ). Fe protein samples were exchanged into a Chelex-100-treated Tris-buffered D_2O solution (70 mg of Tris-HCl and 20 mg of Tris base in 10 mL of D_2O with 4 mM dithionite)

by passage through a Sephadex G-25 column (1.0 × 15 cm). The Fe protein samples were concentrated to between 1 and 2 mM using a Centricon-30 concentrator (Amicon Division, Beverly, MA).

EPR. EPR spectra of dithionite-reduced Fe proteins were recorded at 12 K (Ryle & Seefeldt, 1996). Where indicated, nucleotides were added as a 10-fold molar excess over protein prior to freezing. All spectra were recorded on a Bruker ER300E spectrometer with an Oxford ESR 900 liquid helium cryostat.

Circular Dichroism Spectra of Wild-Type and Ala 98 Altered Fe Proteins. Circular dichroism spectra of indigo-disulfonate- (IDS-) oxidized wild-type and Ala 98 altered Fe proteins were recorded as previously described (Ryle et al., 1996a). Samples of Fe protein (20 mg) were desalted by passage down a Sephadex G-25 column (0.5 × 10 cm) equilibrated with 100 mM Tris buffer, pH 8.0. The Fe protein samples were then oxidized by the addition of 25 μL of 20 mM IDS, and the IDS was removed using a Dowex-1 column. The oxidized Fe protein was diluted to 4 mL and split into two equal samples, and each sample was transferred into sealed 1-cm path-length quartz cuvettes under a nitrogen atmosphere. Nucleotides were added to a final concentration of 1 mM from anaerobic stock solutions. CD spectra were recorded on an Aviv 62DS spectropolarimeter and were baseline subtracted. The concentration of oxidized Fe protein was determined from the absorption at 400 nm using 13 mM^{-1}•cm^{-1} as the absorption coefficient (Lanzilotta et al., 1995b).

Potentiometric Redox Titrations. Potentiometric redox titrations were performed essentially as previously described (Dutton, 1978; Morgan et al., 1986; Ryle & Seefeldt, 1996). All potentials are reported with respect to the normal hydrogen electrode (NHE). Midpoint potentials (E_m) were calculated by fitting the data to the Nernst equation with 1 faraday/mol.

RESULTS

Characterization of the A98G and A98V Altered Fe Proteins. Figure 1 shows a molecular model of the *A. vinelandii* Fe protein based on the X-ray crystal structure (Georgiadis et al., 1992). It is evident that the Fe protein can be described as a three-domain protein, with each subunit as a nucleotide binding domain and the [4Fe-4S] cluster domain as a bridge or hinge domain. Given that it is unlikely that the nucleotide binding sites interact directly, it seems reasonable that communication between the nucleotide binding sites occurs through the [4Fe-4S] cluster domain. The possible role of Ala 98 as a pivot in this domain is apparent in Figure 1. The codon for Ala 98 of the *A. vinelandii* Fe protein gene, *nifH*, was changed to the codons for Gly or Val, and the mutated genes were recombined into the *A. vinelandii* chromosome in place of the wild-type *nifH* gene by a chromosomal gene replacement procedure (Jacobson et al., 1989). The *A. vinelandii* cells expressing either the A98G or A98V altered Fe proteins were tested for their ability to grow under nitrogen-fixing conditions (Seefeldt & Mortenson, 1993). While the cells expressing the A98G altered Fe protein grew at a rate similar to that of wild-type cells, indicating that the A98G altered Fe protein was functional, the cells expressing the A98V Fe protein were unable to grow under nitrogen-fixing conditions. This

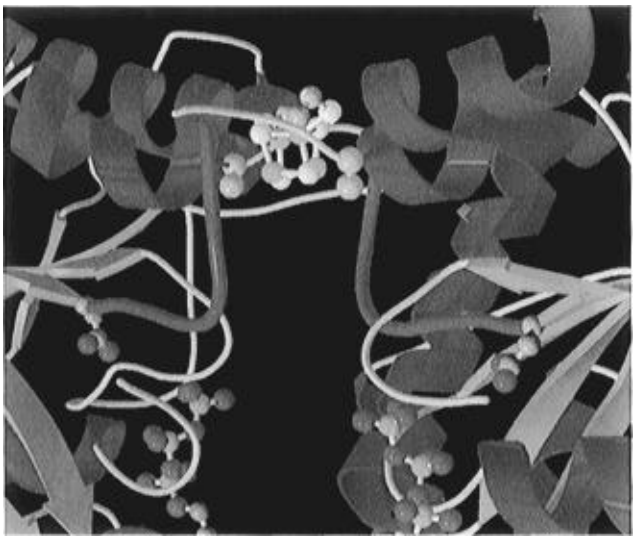

FIGURE 1: Molecular model of the *A. vinelandii* Fe protein. Shown is a close-up view of the [4Fe-4S] cluster domain and a portion of the subunit interface of the Fe protein. The α-carbon trace for each subunit of the homodimer is shown with α-helices in red, β-sheets in green, and coils in yellow. The [4Fe-4S] cluster, which bridges the two subunits, is modeled as a cubane structure near the top of the view with the iron atoms in green and the sulfur atoms in yellow. The two Ala 98 residues (cyan) are shown near the [4Fe-4S] cluster. The two switch II peptide chains (magenta) are shown connecting the [4Fe-4S] cluster to the nucleotide binding domains, with the two Asp 125 residues at the nucleotide binding terminus of switch II. Near the bottom of the view are shown the phosphate portions of the two bound MgATP molecules with phosphorus atoms in green and oxygen atoms in red. The position of the two MgATP molecules was modeled to represent generally where nucleotides are thought to bind and should not be construed to indicate the actual position of binding or the conformational state of the nucleotide-bound protein. The model was generated using the programs MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt & Murphy, 1994) from the X-ray coordinates of the Fe protein (Georgiadis et al., 1992).

suggested that the A98V alteration rendered the Fe protein inactive in nitrogen fixation. In order to probe the functional consequences of these amino acid substitutions, both the A98G and A98V altered Fe proteins were overexpressed in *A. vinelandii* cells, and the altered Fe proteins were purified to homogeneity (Seefeldt & Mortenson, 1993), with yields of 100% and 350%, respectively, of the average wild-type Fe protein yield.

The purified A98G Fe protein was fully active in acetylene reduction, proton reduction, and MgATP hydrolysis activities when combined with the wild-type MoFe protein. In contrast, when the purified A98V Fe protein was combined with the wild-type MoFe protein, no detectable proton reduction, acetylene reduction, or MgATP hydrolysis activities could be detected.

Nucleotide Binding to the Wild-Type and Ala 98 Altered Fe Proteins. The binding of nucleotides (MgATP and MgADP) to the purified A98G and A98V Fe proteins was determined by an equilibrium column binding method previously described (Ryle et al., 1995). From a plot of the ratio of nucleotides bound to the Fe protein versus the concentration of added nucleotide, the number of nucleotides bound, the apparent dissociation constant (K_d), and the magnitude of cooperativity (Hill coefficient) can be determined by fitting the data to the Hill equation. Figure 2 (panel A) shows the data for MgATP binding to the reduced wild-type Fe protein with a calculated apparent K_d of 560 μM

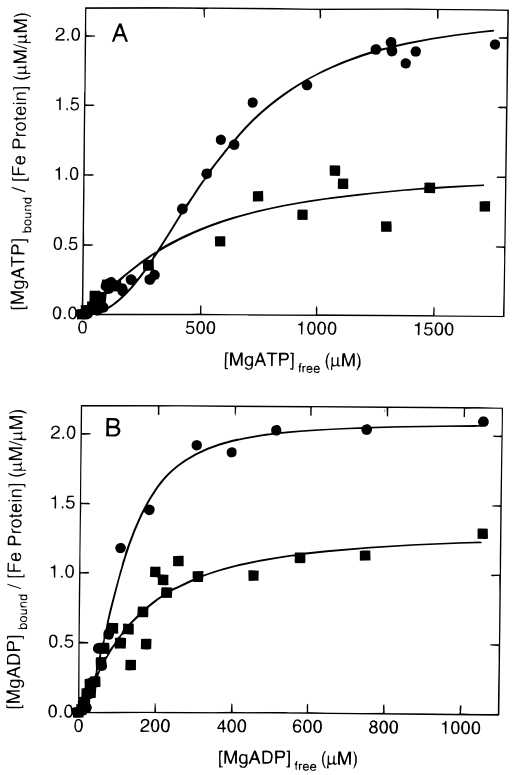

FIGURE 2: Nucleotide binding to wild-type and A98V Fe proteins. Equilibrium binding of MgADP or MgATP to wild-type and A98V Fe proteins was characterized as described in Experimental Procedures. The ratio of the concentration of bound nucleotide divided by the concentration of Fe protein was plotted against the free nucleotide concentration. Data were fit to the Hill equation ($F_b = N_b[S]^n/(K_d + [S]^n)$), where F_b is the fraction of nucleotides bound to the Fe protein, N_b is the maximum number of nucleotides bound per Fe protein, [S] is the free nucleotide concentration, K_d is the apparent dissociation constant, and n is the Hill coefficient (which indicates cooperativity). Panel A: MgATP binding by wild-type Fe protein (●) with a calculated apparent K_d of 560 μM with $n = 2.3$ and $N_b = 2.2$ and by A98V Fe protein (■) with a calculated apparent K_d of 430 μM with $n = 1.2$ and $N_b = 1.1$. Panel B: MgADP binding by wild-type Fe protein (●) with a calculated apparent K_d of 110 μM with $n = 2.0$ and $N_b = 2.1$ and by A98V Fe protein (■) with a calculated apparent K_d of 140 μM with $n = 1.2$ and $N_b = 1.3$.

and with 2.2 bound MgATP molecules with strong positive cooperativity (Hill coefficient $n = 2.3$). These parameters are consistent with previously published results for MgATP binding to *A. vinelandii* Fe protein in the reduced state (Ryle et al., 1995, 1996b; Lanzilotta et al., 1995b). Similar results were obtained for MgATP binding to the A98G altered Fe protein, with an apparent K_d of 480 μM, 2.2 bound MgATP molecules, and strong positive cooperativity ($n = 1.7$). In contrast, under identical assay conditions, the A98V altered protein bound 1.1 MgATP molecules with an apparent K_d of 430 μM and with little cooperativity ($n = 1.2$) (panel A).

Figure 2 (panel B) shows the data for MgADP binding to the reduced state of the wild-type Fe protein. As can be seen, the wild-type Fe protein bound 2.1 MgADP molecules per protein with an apparent K_d of 110 μM and strong positive cooperativity ($n = 2.0$). These parameters are consistent with previously reported values for MgADP binding to *A. vinelandii* Fe protein (Ryle et al., 1996b). Similar binding parameters were determined for MgADP binding to the A98G Fe protein, with 2.0 total bound MgADP molecules with an apparent K_d of 97 μM and strong cooperativity ($n = 2.1$). Again, in contrast, the A98V Fe protein was found to bind 1.3 MgADP molecules with an apparent K_d of 140 μM and little cooperativity ($n = 1.2$) (panel B).

Nucleotide-Induced Conformational Changes in Wild-Type and Ala 98 Altered Fe Proteins. Because the A98V altered Fe protein bound 1 MgATP or 1 MgADP compared to the binding of two nucleotides to the wild-type and A98G Fe proteins, it was of interest to determine the nature and extent of protein conformational changes that occurred in the A98V Fe protein upon binding of one nucleotide. The extent of the nucleotide-induced conformational changes in the A98V Fe protein was monitored using a number of methods, including Fe chelation rate, EPR, CD, and NMR spectroscopies, and changes in the midpoint potential (E_m).

One sensitive assay to monitor protein conformational changes in the Fe protein is the MgATP-dependent chelation of Fe^{2+} from the Fe protein by chelators such as bathophenanthrolinedisulfonic acid (BPS) (Ljones & Burris, 1978). The wild-type and A98G Fe proteins showed apparent first-order rates of Fe chelation to BPS only upon the addition of MgATP, with apparent first-order rate constants of 0.027 and 0.029 s^{-1}, respectively. Likewise, the A98V Fe protein only showed Fe chelation to BPS upon the addition of MgATP, but the apparent first-order rate constant was significantly lower (0.005 s^{-1}). In order to determine if the lower rate of Fe chelation from the A98V Fe protein was the result of a lower total Fe content (i.e., incomplete [4Fe-4S] clusters), the total Fe content of the wild-type, A98G, and A98V Fe proteins was determined by an Fe analysis assay (Seefeldt et al., 1992) and found to be 3.41 ± 0.25 Fe per wild-type Fe protein, 3.51 ± 0.24 Fe per A98G Fe protein, and 3.60 ± 0.17 Fe per A98V Fe protein. Thus, the lower rate of iron chelation from the A98V Fe protein was not the result of a lower total iron content.

EPR. The line shape of the EPR spectrum of reduced Fe protein is sensitive to the nucleotide bound state (Zumft et al., 1974; Lindahl et al., 1987). The binding of MgADP or MgATP to the Fe protein results in different line shape changes, the most pronounced change being from rhombic to axial upon binding MgATP. EPR spectra for wild-type, A98G, and A98V altered Fe proteins, both in the absence and presence of MgATP or MgADP, are shown in Figure 3. In the absence of nucleotides, all three proteins showed rhombic EPR line shapes (Figure 3, panel A). Subtle changes in some *g*-values were observed for the A98V altered Fe protein, suggesting minor perturbations of the electronic structure of the [4Fe-4S] cluster resulting from the amino acid change. The addition of MgATP to the wild-type and A98G Fe proteins resulted in the expected line shape change to axial (panel B). In contrast, the A98V Fe protein showed little change in EPR line shape upon the addition of MgATP. Likewise, the wild-type and A98G Fe proteins, but not the A98V Fe protein, showed the expected changes in EPR spectral line shape upon MgADP addition. The EPR spectrum of the A98G Fe protein with MgADP bound is significantly broadened, which could indicate increased conformational flexibility around the [4Fe-4S] cluster by changing to Gly at this position.

Proton NMR. The downfield portion of the proton NMR spectrum of the reduced Fe protein contains resonances from the eight β-CH_2 and four α-CH protons of the four cysteinyl ligands to the [4Fe-4S] cluster, which appear as four distinct resonances between 13 and 50 ppm (Lanzilotta et al., 1995a).

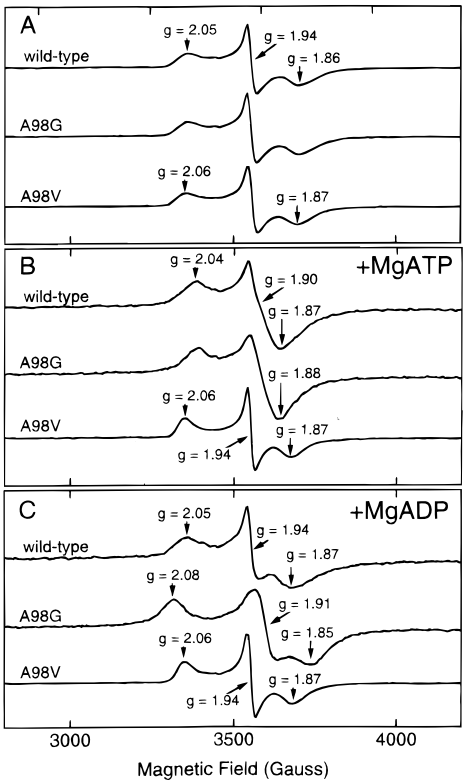

FIGURE 3: EPR spectra of wild-type and Ala 98 altered Fe proteins in the absence or presence of nucleotides. Wild-type, A98G, and A98V Fe proteins (30 mg•mL^{-1}) were prepared, and spectra were recorded, as described in Experimental Procedures. Samples were maintained in the reduced state by the presence of 2 mM dithionite. Spectra were recorded at a temperature of 12 K, a frequency of 9.64 GHz, and a microwave power of 1.0 mW. EPR spectra are presented for wild-type, A98G, and A98V Fe proteins in the absence of nucleotides (panel A), with a 10-fold molar excess of MgATP (panel B), and with a 10-fold molar excess of MgADP (panel C).

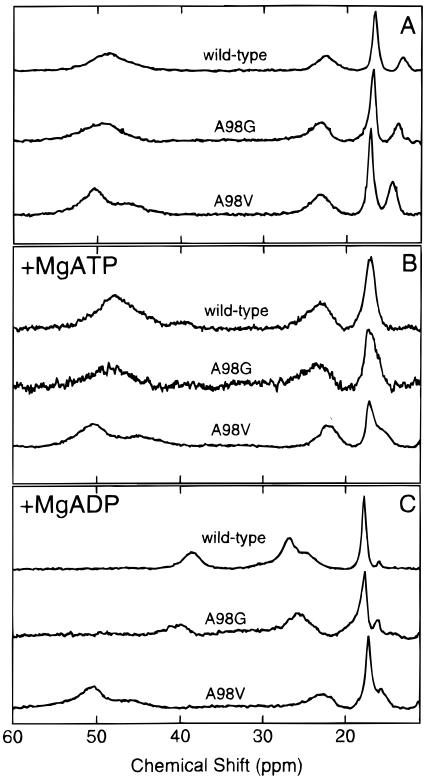

FIGURE 4: Downfield portion of the ^{1}H NMR spectra of wild-type and Ala 98 altered Fe proteins. Reduced Fe protein samples were prepared as described in Experimental Procedures. The downfield portion of the 400.17 MHz ^{1}H NMR spectrum, at a temperature of 305 K, is presented for wild-type (1.5 mM), A98G (1 mM), and A98V (1.7 mM) Fe proteins in the absence of nucleotides (panel A), in the presence of MgATP (panel B), and in the presence of MgADP (panel C). Nucleotides were added from stock solutions to a 5-fold molar excess over the protein concentration.

The chemical shifts of these resonances are sensitive to the distance and orientation of the cysteinyl ligands relative to the [4Fe-4S]$^{1+}$ cluster. The binding of MgADP or MgATP by the wild-type Fe protein results in changes in the isotropic shifts of these resonances, which supports the model that the binding of nucleotides by the Fe proteins induces changes in the [4Fe-4S] cluster environment (Lanzilotta et al., 1995a). The 400 MHz ^{1}H NMR spectra for wild-type and Ala 98 altered Fe proteins in the absence or presence of nucleotides are presented (Figure 4). In the absence of any nucleotides, the downfield proton NMR spectra for all three proteins were nearly identical (panel A). The A98V alteration did result in a shift of the 49 ppm resonance to 51 ppm and in the resolution of this resonance into two overlapping signals. This signal has been assigned to two β-CH_2 cysteinyl proton pairs bound to the mixed valence iron pair of the cluster (Lanzilotta et al., 1995a).

Changes in the downfield proton NMR resonances of the three Fe proteins upon nucleotide binding are also shown (panels C and D). Both the wild-type and A98G Fe proteins showed nearly identical spectra in the presence of MgATP or MgADP. In contrast, the A98V showed only minor changes in NMR signals upon binding nucleotides when compared to the no nucleotide bound state. Only the 14 ppm resonance of the A98V Fe protein, which has been assigned to protons of an α-CH cysteinyl ligand bound to the all ferrous Fe pair (Lanzilotta et al., 1995a), showed any change in chemical shift upon nucleotide addition. These observations suggest that the A98V Fe protein undergoes a partial conformational change upon binding one nucleotide.

Circular Dichroism. The most sensitive spectroscopic technique for monitoring the effects of nucleotide binding on the properties of the [4Fe-4S]$^{2+}$ cluster is the visible region circular dichroism (CD) spectrum (Stephens et al., 1979; Ryle et al., 1996a). It has recently been shown that the no nucleotide, MgATP- and MgADP-bound states of the Fe protein give distinct CD spectra, providing a fingerprint for these three states (Ryle et al., 1996a). The CD spectra for the wild-type, A98G, and A98V Fe proteins in the absence of nucleotides are shown in Figure 5 (panel A). Both the wild-type and A98G Fe proteins gave CD spectra consistent with previously reported data (Ryle et al., 1996a). The A98V Fe protein showed a somewhat different CD spectrum, most notably at 450 and 370 nm. The addition of MgATP to all Fe proteins resulted in CD spectra similar to that previously reported for the MgATP-bound state of the wild-type Fe protein (panel B). Most interesting was the effect of adding MgADP (panel C). While the wild-type and A98G Fe proteins gave spectra that are expected for the MgADP-bound state, the A98V Fe protein gave a spectrum that was dramatically different, looking more like the spectrum observed for wild-type Fe protein with MgATP bound.

Redox Potentials. One important change in the properties of the [4Fe-4S] cluster upon the binding of MgATP or

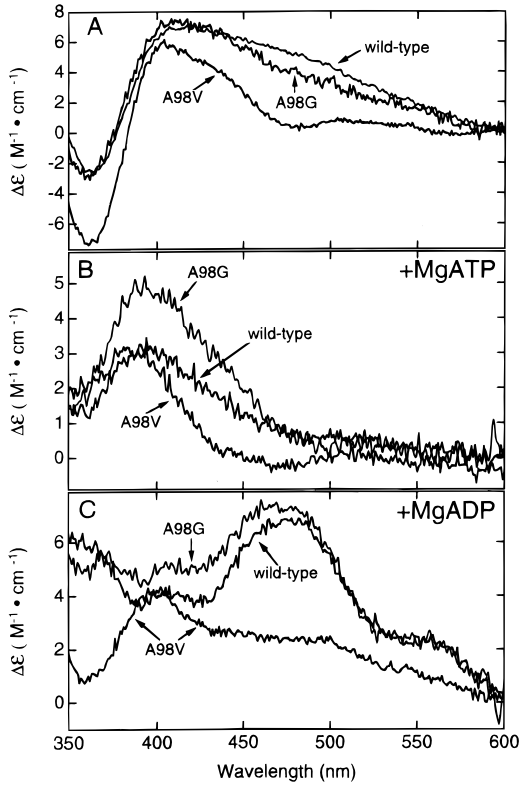

FIGURE 5: Circular dichroism spectra of wild-type and Ala 98 altered Fe proteins in the absence or presence of nucleotides. The visible region circular dichroism (CD) spectra of IDS-oxidized wild-type, A98G, and A98V altered Fe proteins are presented in the absence of nucleotides (panel A), in the presence of 1 mM MgATP (panel B), and in the presence of 1 mM MgADP (panel C). All spectra were baseline subtracted, and the presence of the oxidized state was confirmed from the visible absorption spectrum.

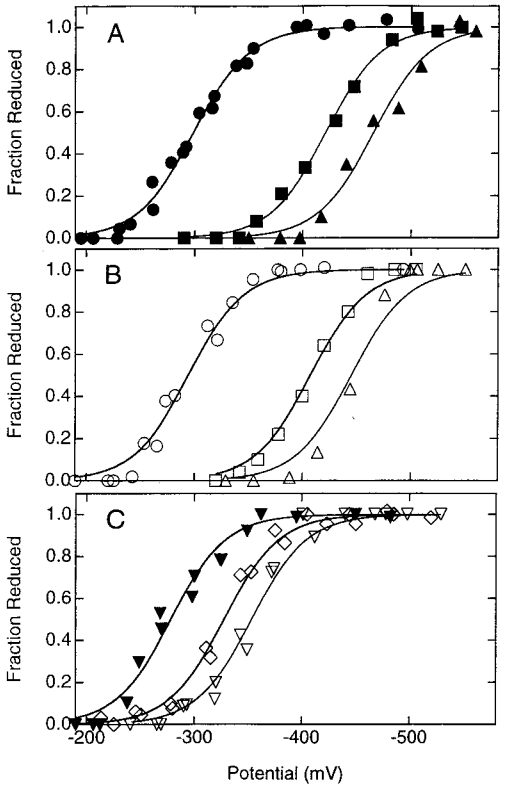

FIGURE 6: Redox titrations of wild-type and Ala 98 altered Fe proteins. Potentiometric titrations of Fe protein samples (10−15 mg·mL^{-1}) were done in 50 mM Tricine buffer, pH 8.0, with 250 mM NaCl, 50 μM methyl viologen, 50 μM benzyl viologen, and 50 μM flavin mononucleotide. Panel A: Potentiometeric titrations of wild-type Fe protein without added nucleotides (●, $E_m = -300$ mV), with MgATP (■, $E_m = -420$ mV), or with MgADP (▲, $E_m = -460$ mV). Panel B: Potentiometeric titrations of A98G Fe protein without added nucleotides (○ , $E_m = -290$ mV), with MgATP (□, $E_m = -410$ mV), or with MgADP (▲, $E_m = -450$ mV). Panel C: Potentiometeric titrations of A98V Fe protein without added nucleotides (▼, $E_m = -280$ mV), with MgATP (▽, $E_m = -360$ mV), or with MgADP (◇, $E_m = -330$ mV). All EPR spectra were recorded at a temperature of 12 K, a microwave power of 6.36 mW, and a frequency of 9.64 GHz. Fits of the data to the Nernst equation with 1 faraday/mol are shown. Midpoint potentials (E_m) are reported relative to the normal hydrogen electrode (NHE). Nucleotides were added to 5 times the protein concentration.

MgADP to the Fe protein is the change in the redox potential of the 2+/1+ couple from −300 mV to −420 or to −460 mV, respectively (Zumft et al., 1974; Watt et al., 1986). Figure 6 shows potentiometric titrations for wild-type (panel A), A98G (panel B), and A98V (panel C) Fe proteins in the absence of nucleotides, in the presence of MgATP, and in the presence of MgADP. The calculated midpoint potentials (E_m) from fits of the data to the Nernst equation are summarized in Table 1. The wild-type and A98G Fe proteins gave similar E_m values in all cases. Likewise, the A98V Fe protein showed the expected E_m in the absence of nucleotides. Surprisingly, however, the A98V Fe protein showed only approximately half the shift in midpoint potential in the presence of MgATP or MgADP. These results, coupled with the results from the spectroscopic analyses, suggest that binding of a single nucleotide to the A98V Fe protein induces only a portion of the protein conformational changes found for the binding of two nucleotides to the wild-type Fe protein.

Binding of the A98V Fe Protein to the Wild-Type MoFe Protein. Because the A98V Fe protein was inactive in MgATP hydrolysis, yet remains able to bind nucleotides and still undergoes a portion of the nucleotide-dependent conformational changes, it was of interest to determine if this protein could still dock to the MoFe protein. The docking of the Fe protein to the MoFe protein involves a complex series of events, where the MgATP-bound state of the Fe protein is required for the final stages of docking and the MgADP-bound state for the dissociation of the component proteins. To address the potential affinity of the A98V Fe protein for docking to the MoFe protein, a competitive binding assay was employed (Lanzilotta et al., 1995b). The A98V Fe protein was observed to inhibit the association of the wild-type Fe protein with the MoFe protein during a normal activity assay, with a 50% inhibition of activity at a molar ratio of 17 A98V Fe proteins to 1 MoFe protein (Figure 7). Thus, the A98V Fe protein is still able to bind to the MoFe protein, although the complex is not competent for MgATP hydrolysis or electron transfer.

DISCUSSION

The results of the present study can be discussed in the context of three important questions concerning the function of the nitrogenase Fe protein: (i) What is the mechanism of cooperative binding of two nucleotides by the Fe protein, (ii) how does nucleotide binding induce changes in the properties of the [4Fe-4S] cluster (e.g., changes in the midpoint potential), and (iii) what is the role of nucleotide-induced protein conformational changes in component protein docking, MgATP hydrolysis, and intercomponent electron transfer?

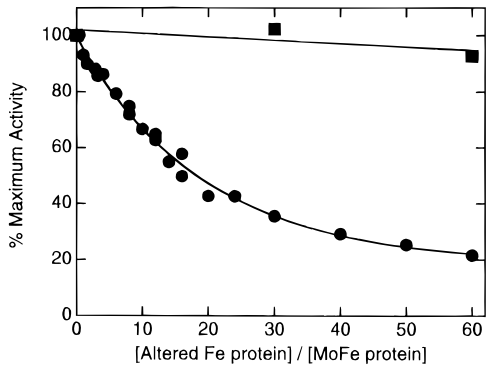

FIGURE 7: Inhibition of wild-type acetylene reduction activity by the A98V Fe protein. Acetylene reduction assays were performed as described in Experimental Procedures. A constant amount of wild-type MoFe protein (1.0 nmol) and increasing amounts of A98V (●) or K15R (■) Fe proteins (0−61 nmol) were added to each assay vial. Each reaction was initiated by the addition of 2.0 nmol of wild-type Fe protein. The percentage of the maximum acetylene reduction activity (nanomoles of C_2H_4 formed per minute) was plotted against the ratio of altered Fe protein divided by the wild-type MoFe protein. The maximum activity of the wild-type Fe protein−MoFe protein complex was 2050 nmol of C_2H_4 formed·min^{-1}·(mg of Fe protein)$^{-1}$.

Table 1: Redox Potentials of Wild-Type and Ala 98 Altered Fe Proteins

	E_m (mV)[a]					
addition	WT Fe protein	ΔE_m[b]	A98G Fe protein	ΔE_m	A98V Fe protein	ΔE_m
no addition	−300		−290		−280	
MgATP	−420	−120	−410	−120	−360	−80
MgADP	−460	−160	−450	−160	−330	−50

[a] Midpoint potentials (E_m) are relative to the normal hydrogen electrode (NHE). [b] Changes in the midpoint potentials (ΔE_m) upon the addition of the noted nucleotide.

Mechanism of Cooperative Nucleotide Binding by the Fe Protein. The site and amino acids involved in binding the phosphate portion of nucleotides to the Fe protein are reasonably well defined. From the location of a bound ADP in the crystal structure of the *A. vinelandii* Fe protein (Georgiadis et al., 1992) and the analysis of a series of site-specifically altered Fe proteins, the phosphate portion of each bound nucleotide appears to be located near the subunit interface, about 19 Å below the [4Fe-4S] cluster. Lys 15 appears to make important electrostatic interactions with the β- and γ-phosphates (Seefeldt et al., 1992; Ryle et al., 1995), while Asp 125 (Wolle et al., 1992a) and Ser 16 (Seefeldt & Mortenson, 1993) appear to coordinate the bound Mg^{2+}, either directly or through bound waters. Asp 129 also appears to interact with the phosphates, again probably through a bound water (Lanzilotta et al., 1995b). The locations of the ribose and adenine portions of bound nucleotides are not clear, with two very different models having been suggested (Wolle et al., 1992a). Two important questions are: (i) how is the energy of nucleotide binding communicated to the [4Fe-4S] cluster, which is observed as changes in the properties of the [4Fe-4S] cluster, and (ii) how is nucleotide binding to one subunit of the Fe protein communicated to the other subunit. The peptide chains from Asp 125 to Cys 132 (designated switch II) have been shown to be one pathway for communication from the nucleotide binding site to the [4Fe-4S] cluster (Ryle & Seefeldt, 1996). It was suggested that the binding of nucleotides results in a pull on switch II, leading to changes in the protein environment around the [4Fe-4S] cluster and hence to changes in its properties. The results of the present study extend the previous model by suggesting that the [4Fe-4S] cluster domain of the Fe protein is an intermediate in the transduction from one nucleotide binding site to the other in the cooperative binding mechanism. It might be imagined that nucleotide binding to one subunit of the Fe protein results in movement in switch II within that subunit. The resulting changes in the properties of the [4Fe-4S] cluster domain could result in movement in switch II within the other subunit, transducing a signal to the second nucleotide binding site. Additional support for such a model comes from several previous observations. Changing Phe 135, located near the [4Fe-4S] cluster, to Trp or His resulted in proteins with a significantly higher affinity for binding nucleotides (Ryle et al., 1996b). Hence, changes near the [4Fe-4S] cluster domain were communicated to the nucleotide binding sites. Likewise, reaction of the [4Fe-4S] cluster with NO resulted in an Fe protein that no longer bound nucleotides (Hyman et al., 1992). Finally, the redox state of the [4Fe-4S] cluster also appears to regulate the affinity for binding nucleotides, with the oxidized (2+) state having a significantly higher affinity for binding both MgATP and MgADP when compared to the reduced (1+) state (Cordewener et al., 1985; Yates, 1991; Ryle et al., 1996b).

This raises the obvious question as to why two nucleotides bind to the Fe protein? The answer may well lay in the energetics of nucleotide binding. The free energy of nucleotide binding (ΔG) must be used to drive the protein conformational changes that ready the Fe protein for docking to the MoFe protein and to drive the lowering of the redox potential. The free energy change for two nucleotides binding would provide twice the energy, which may be required for these conformational changes. The other answer may be in the energetics of MgATP hydrolysis. It has been suggested that the energy of MgATP hydrolysis by the Fe protein when bound to the MoFe protein drives the transfer of an electron between the proteins or to substrates. Again, the hydrolysis of two MgATP molecules would provide twice the energy liberated from the hydrolysis of a single nucleotide (30 kJ·mol^{-1}). This extra energy may be required to overcome the significant activation energy for the reduction of dinitrogen. This logic suggests a reason for cooperativity in the binding of two nucleotides to the Fe protein. Positive cooperativity ensures that if one nucleotide is bound, then there is a very high probability that the second will also be bound. This would prevent the formation of a catalytically inactive, partially ligated Fe protein.

Nucleotide-Induced Changes in the Properties of the [4Fe-4S] Cluster. Three models seem likely to explain the nucleotide-induced changes in the protein conformation of the Fe protein: (i) The binding of the first nucleotide to the Fe protein could induce all of the conformational changes in the protein, (ii) the binding of the second nucleotide could induce all of the changes, or (iii) the binding of each nucleotide could contribute to the total conformational change. The results from the present work demonstrate that the A98V Fe protein binds only one nucleotide and appears to undergo only about half the conformational changes, supporting the third model above. As might be expected, the binding of each nucleotide contributes to the conformational changes.

The Role of Nucleotide-Induced Conformational Changes in Nitrogenase Catalysis. The A98V Fe protein described in this work provides a unique opportunity to determine the role of the stoichiometry of nucleotide binding and the resulting conformational changes on the catalytic functions of the Fe protein. Of particular interest would be the need for one or two nucleotides to induce protein conformational changes in the Fe protein required for docking to the MoFe protein, activation of MgATP hydrolysis in the protein complex, and activation of intercomponent electron transfer. The observation that the A98V Fe protein still bound to the MoFe protein, but did not hydrolyze MgATP or transfer electrons, suggests that the partial conformational changes that occur in the A98V Fe protein are sufficient to allow some stage of docking to the MoFe protein, but not MgATP hydrolysis or electron transfer. It is tempting to ascribe the lack of electron transfer from the A98V Fe protein to the MoFe protein to the more positive redox potential of the cluster in the nucleotide-bound state. This possibility seems unlikely, however, because the Fe protein from the bacterium *Klebsiella pneumoniae* has a significantly more positive redox potential in the MgATP-bound state (−320 mV) (Ashby & Thorneley, 1987) compared to that of the *A. vinelandii* Fe protein in the MgATP bound state (−420 mV), and yet the *K. pneumoniae* Fe protein transfers an electron to the *A. vinelandii* MoFe protein at the same rate as the *A. vinelandii* Fe protein.

Comparison to Other Proteins Which Cooperatively Bind Substrates. The cooperative binding of nucleotides by the Fe protein, and the proposed function of the [4Fe-4S] cluster in this mechanism, can be compared to other proteins that bind ligands cooperatively. Similar to many allosteric proteins, the Fe protein contains multiple subunits, with ligands binding at different subunits and cooperativity mediated between the subunits. Unlike any other known examples of allosteric proteins, however, the [4Fe-4S] cluster domain of the Fe protein appears to play a pivotal role in the cooperative mechanism. The classic example of an allosteric protein is hemoglobin. In this protein, the binding of a single oxygen to a heme of one subunit results in changes in the affinity for binding oxygen to hemes in other subunits (Ackers & Hazzard, 1993). This cooperativity is mediated by a 1 Å movement of the axial ligated histidine, which is propagated to the subunit interface. This results in a 200-fold higher affinity for the binding of oxygen to the other subunits.

Single amino acid substitutions have been used in several other allosteric proteins to probe the mechanism of cooperative ligand binding. For example, calmodulin binds two Ca^{2+} ions cooperatively, one to each of the EF binding domains (Linse et al., 1991). Asp 95 of the C-terminal domain has been identified as one of the ligands to the bound Ca^{2+}, and altering this residue to Asn decreased the affinity for binding Ca^{2+} and decreased the cooperativity for binding the second nucleotide (Waltersson et al., 1993). When a homologous alteration was made in the N-terminal EF binding domain (D48N), both the affinity and cooperativity for binding calcium were increased. Muscle glycogen phosphorylase is a homodimeric protein which binds two MgAMP molecules with strong positive cooperativity (Sprang et al., 1987, 1988). This enzyme is similar to the Fe protein in that each nucleotide binding site is located within one of two identical subunits. However, unlike the Fe protein, in which the subunits are linked through the [4Fe-4S] cluster domain, the subunits of glycogen phosphorylase are linked through several peptide chains. Recently, site-directed mutagenesis studies have been carried out within these peptide chains. The alteration of Pro 48, a residue located within the peptide bridges between the individual subunits, to Thr resulted in a change in the binding of two MgAMP molecules from strongly cooperative to noncooperative (Browner et al., 1992). The structural analysis of the P48T altered protein showed no major atomic rearrangement at the MgAMP binding sites; hence, the alteration was suggested to interfere with the transmission of the allosteric signal (Browner et al., 1992). Although no structural information concerning the nucleotide binding sites of the A98V Fe protein is available, it seems unlikely that the alteration of Ala 98 has resulted in direct perturbation of the nucleotide binding site. Therefore, much like the P48T alteration in muscle glycogen phosphorylase, the A98V alteration in the Fe protein probably also interferes with allosteric signal transduction.

In summary, the present work provides evidence that the conserved Ala at position 98 in the nitrogenase Fe protein plays an important role in communicating the conformational changes which are required for the cooperative binding of two nucleotides. It also appears that the binding of a single nucleotide by the A98V Fe protein induces about half the changes in the physicochemical properties of the [4Fe-4S] cluster that are expected for the binding of two nucleotides. In light of these observations, the [4Fe-4S] cluster domain can be viewed as a hinge between the two nucleotide binding domains which facilitates conformational rearrangements required for the cooperative binding of a second nucleotide.

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