Structure-Based Mutational and Functional Analysis Identify Human NM23-H2 as a Multifunctional Enzyme†

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Received January 30, 2002; Revised Manuscript Received March 6, 2002

ABSTRACT: The human NM23-H2 protein is a transcriptional regulator (PuF) that binds and cleaves DNA via covalent bond formation, and also catalyzes phosphoryl transfer (NDP kinase). Our previous work has identified two separate DNA-binding regions on NM23-H2/PuF: a sequence-dependent DNA-binding surface involving residues Arg34, Asn69, and Lys134 on the equator of the hexameric protein and a covalent DNA-binding site involving Lys12 located in the nucleotide-binding site, the site of the NDP kinase reaction. To understand the role of the nucleotide-binding site in the DNA cleavage reaction and to establish a connection between the nuclease and the NDP kinase activities, we used the known crystal structure of NM23-H2 complexed with GDP as the basis for site-directed mutagenesis. We thus identified Arg88 and Arg105 as residues that are, in addition to Lys12, critical for covalent DNA binding and DNA cleavage, as well as for the NDP kinase reaction. Another residue, Gln17, was required only for DNA cleavage, and Tyr52, Asn115, and His118 were found to be essential only for the NDP kinase activity. Six of these seven functionally important amino acids associated with the nucleotide-binding site are evolutionarily conserved, underscoring their biological importance. We also show that nucleoside triphosphates but not nucleoside diphosphates inhibited the covalent DNA binding and DNA cleavage reactions, independent of phosphoryl transfer and the NDP kinase reaction. These findings collectively suggest that the binding modes of mononucleotides and duplex DNA oligonucleotides in the nucleotide-binding site differ, and that NM23-H2 possesses multiple biochemical activities. A model consistent with these observations is presented.

NM23-H2 belongs to a large family of highly conserved proteins known for their roles in development and cancer, but how these proteins contribute to their various physiological and pathological functions is unclear. For decades, NM23 proteins have been characterized by enzymatic means as NDP[1] kinases (EC 2.7.4.6), and we now have a detailed understanding of the kinetic reactions they catalyze. This involves the transfer of a γ-phosphoryl group from nucleoside triphosphates to nucleoside diphosphates through a ping-pong mechanism that uses a conserved histidine residue as an intermediate, where the imidazole side chain becomes autocatalytically phosphorylated. NDP kinases are highly efficient enzymes that are able to utilize purine or pyrimidine, ribo- or deoxyribonucleoside triphosphates and diphosphates as substrates (reviewed in ref *1*). In X-ray structures, the 145−152-amino acid length subunits consist of a fold of identical four-stranded antiparallel β-sheets surrounded by α-helices, an unusual fold for phosphotransferases (*2*−*4*). Dimerization of the subunits takes place via interactions between antiparallel α-helices and a continuous β-strand. The eukaryotic enzymes are hexameric, while most of the bacterial enzymes form tetramers. NM23/NDP kinases lack a classical mononucleotide binding fold and a P-loop, and contacts with the base moiety are not specific (reviewed in ref *5*).

NDP kinases were originally described as essential housekeeping enzymes required for the synthesis of nucleoside triphosphates, and for the general maintenance of nucleotide pools. However, in *Escherichia coli* and in yeast, NDP kinase is not essential for viability, although deletion of the *ndk* gene in *E. coli* produces a phenotype suggestive of DNA repair defects (*6*, *7*), and a knockout strain in *Saccharomyces pombe* exhibits developmental abnormalities (*8*). In *Drosophila*, the *awd*/*ndpk* gene is required for late larval development (reviewed in ref *9*). The human NM23/NDPK family consists of eight related genes and widely expressed proteins termed NM23-H1−NM23-H8, some of which play critical roles in the control of development (*10*−*13*). NM23-H1 and NM23-H2, the two most abundant and closely related human isoforms, have been shown to play significant roles in the genesis and metastasis of some cancers (reviewed in refs *14* and *15*).

† This work was supported by NIH/NCI Grant CA76496 to E.H.P.

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[1] Abbreviations: NM, nonmetastatic; NDP, nucleoside diphosphate; BER, base excision repair; NHE, nuclease-hypersensitive element; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EMSA, electrophoretic mobility shift assay; TBE, Tris-borate-EDTA.

10.1021/bi025606+ CCC: $22.00
Published on Web 04/27/2002

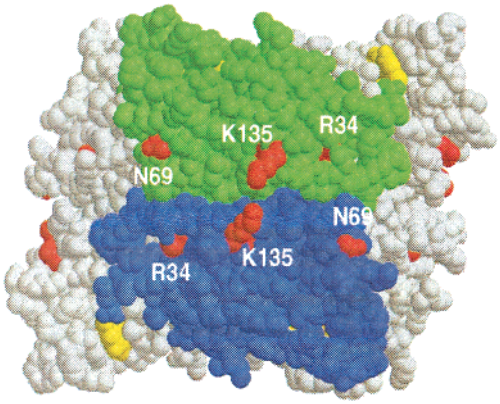

FIGURE 1: Space-filling model of the NM23-H2/NDP kinase B hexamer as shown in a complex with GDP (yellow) (from ref *3*). The sequence-dependent DNA-binding residues are shown in red (from ref *21*). See the text for details.

Earlier studies in our laboratory have identified human NM23-H2 as PuF, a HeLa cell factor that binds DNA and activates transcription via unusually structured c-*MYC* promoter DNA elements (*16*−*18*). Biochemical and mutational studies established the phosphorylation site of NM23-H2 as His118 (*19*, *20*), a residue located in the nucleotide-binding site. A mutational analysis of sequence-dependent DNA binding identified a binding surface on the equator of the hexameric protein as involving Arg34, Asn69, and Lys135 (Figure 1; *21*). It was concluded on the basis of these studies (*20*, *21*) that sequence-dependent DNA binding and in vitro transcriptional activation are properties independent from His118 phosphorylation and, conversely, that the NDP kinase reaction does not require sequence-dependent DNA binding, altogether suggesting that NM23-H2 might be bifunctional (*20*, *21*).

More recently, we have identified a second enzymatic activity associated with NM23-H2 that involves the reversible cleavage of DNA through a covalent interaction with Lys12, a residue located in the nucleotide-binding site (*7*, *22*). As suggested by a sodium borohydride-dependent enzyme−DNA cross-linking assay, the reaction between DNA and Lys12 involves Schiff base formation, where the enzyme is covalently bonded to DNA (*7*). These findings implied the existence of a second DNA-binding region on NM23-H2 located in the nucleotide-binding site. Because DNA cleavage initially requires sequence-dependent DNA binding, we surmised that the surface of the equator is used by the enzyme for positioning the DNA substrate into the nucleotide-binding site for the cleaving reaction (*7*; Figure 1). As the nucleotide-binding site is the reactive site of NDP kinase transphosphorylation, our observations suggested a connection between the two seemingly disparate enzymatic activities, NDP kinase and the nuclease. Furthermore, because a lysine-based DNA cleavage chemistry is the signature mechanism of bifunctional DNA glycosylase/lyase base excision repair (BER) enzymes, these findings also raised the possibility that NM23-H2 may be involved in DNA repair (*7*).

In this report, we sought to study the catalytic reaction of the nuclease by identifying functionally important amino acid residues, and by determining the extent to which the DNA cleavage and NDP kinase enzymatic activities are integrated. On the basis of mutational and functional analysis, we demonstrate here that the nucleotide-binding site plays a critical role in not only the NDP kinase reaction but also the reactions involving covalent DNA binding and DNA cleavage catalysis. We also show that nucleoside triphosphates exert a dramatic effect on the DNA-related activities. In the context of the known NM23-H2 crystal structure−GDP complex (*3*), we suggest that the modes of binding of mononucleotides and duplex DNA oligonucleotides to the nucleotide-binding site differ. Modeling of the important amino acid residues involved in these reactions suggests that NM23-H2 possesses multiple but related biochemical activities.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Some of the mutants used in these studies have already been described: T7V, K12Q, K12R (*7*), R34A, Y52V, R88A, P101S, R105A (*21*), and H118F (*20*). The oligonucleotides used to generate the new mutations were as follows (from the 5′- to 3′-end, with base changes indicated with bold letters): K12A, CGCACCTTCATCGCCATC**GCG**CCGGACGGCGTGCAGCGC; P13G, CGCACCTTCATCGCCATCAAG**GGG**GACGGCGTGCAGCGC; D14E, CGCACCTTCATCGCCATCAAGCCG**GAG**GGCGTGCAGCGC; Q17N, GCCATCAAGCCGGACGGCGTG**AAC**CGCGGCCTGGTGGGC; Y67F, CCATTCTTCCCTGGGCTGGTGAAG**TT**CATGAACTCAGGGCCG; N115D, CAGGTTGGCAGG**GAC**ATCATTCATGGC; S122P, CATGGCAGTGAT**CCA**GTAAAAAGTGCTGAA; and W149F, GACTACAAGTCTTGTGCTCATGAC**TTC**GTCTATGAA.

The desired mutations and the absence of unwanted changes were verified by sequence analysis of the entire coding region. Proteins were expressed in *E. coli* strain BL21(DE3) and purified as described previously (*21*).

Oligonucleotide Substrates Used for DNA Cleavage Analyses. The oligonucleotides used for cleavage assays were a 45 bp synthetic duplex with the NHE sequence (top strand) 5′-AGTCTCCTCCCCACCTTCCCCACCCTCCCCACCCTCCCCATAAGC-3′ and a 34 bp synthetic duplex with the NHE sequence (top strand) 5′-CTCCCCACCTTCCCCACCCTCCCCACCCTCCCCA-3′. They were made double-stranded by annealing the complementary strands, 5′-^{32}P-end-labeled using T4 polynucleotide kinase and [γ -^{32}P]-ATP, and purified by PAGE as previously described (*22*).

Assay for Covalent Complex Formation. Covalent protein−DNA complexes were analyzed by SDS−PAGE as described previously (*22*). Briefly, uniformly ^{32}P-labeled plasmid DNA containing the c-*MYC* NHE sequence was mixed with NM23-H2 proteins and incubated in reaction buffer [50 mM Tris-HCl (pH 8.0), 120 mM KCl, 50 μg/mL BSA, 0.1 mM EDTA, 1 mM dithiothreitol, and 1.5 mM $MgCl_2$] at 25 °C for 30 min. The reaction was terminated by heating for 10 min at 90 °C, and the bulk DNA was digested with DNase and exonuclease III. The remaining protein−oligonucleotide complexes were precipitated with 10% TCA at 0 °C, boiled in SDS−PAGE sample buffer for 5 min, and then resolved on 4 to 15% SDS−PAGE gels. Gels were fixed and vacuum-dried prior to exposure to X-ray film.

Gel Mobility Shift Assay (EMSA) for DNA Binding and DNA Cleavage Analysis. Reaction mixtures (20 μL) were assembled in reaction buffer containing 250−500 ng of each

Table 1: DNA Cleavage, Covalent DNA Binding, NDP Kinase Activity, and Oligomerization State of NM23-H2 Mutants

protein	cleaved DNA (%)[a]	covalent complex[b]	NDPK specific activity (%)[c]	oligomerization state[d]
wild type	100	+	100	hexamer
T7V	90 ± 9.5	+	68 ± 8.2	nt
K12A[e]	1.7 ± 1.3	−	−	nt
K12Q	1.6 ± 1.3	−	−	hexamer
K12R	30 ± 5.5	+	13 ± 3.6	nt
P13G	30 ± 5.5	+	45 ± 6.7	hexamer
D14E	44 ± 6.6	+	42 ± 6.5	hexamer
Q17N	1.0 ± 1	+	97 ± 9.9	nt
Y52V	110 ± 10.5	+	−	hexamer
Y67F	100 ± 10	+	100 ± 10	nt
S70A	100 ± 10	+	83 ± 9.1	nt
R88A	−	−	−	hexamer
P101S	90 ± 9.5	+	91 ± 9.5	hexamer
R105A	1.3 ± 1.1	−	−	hexamer
N115D	84 ± 9.2	+	−	hexamer
H118F	110 ± 10.5	+	−	hexamer
S120G	90 ± 9.5	+	76 ± 8.7	nt
S122P	90 ± 9.5	+	93 ± 9.6	nt
W149F	80 ± 8.9	+	100 ± 10	nt

[a] Percentage of DNA cleaved by a mutant relative to wild-type protein in EMSAs. A dash (−) indicates no detectable activity. [b] Wild-type levels of covalent complexes formed in SDS−PAGE gels are indicated with a plus (+); decreases of ≥50-fold are indicated by a dash (−). [c] The specific NDP kinase activity of each mutant is expressed as a percentage of wild-type activity (731 units/mg). These values represent the average of three independent determinations. A dash (−) indicates no detectable activity. [d] As determined by size exclusion chromatography. Proteins that were not tested (nt) were assumed to be hexameric on the basis of either their normal NDP kinase activities or cleavage beavior. [e] Mutants designated in bold letters had no detectable activity with respect to one of three criteria.

protein and 25 fmol of labeled oligonucleotide substrates. Samples were incubated on ice for 20 min, and the reaction products were separated on 5% polyacrylamide gels in 1× TBE buffer for 35 min at 100 V and 25 °C. The gels were dried and exposed to XAR film, and the amount of DNA cleaved was estimated by phosphorimaging (Molecular Dynamics). At least three separate EMSAs were performed using at least two independently purified protein samples. The results were averaged and expressed as a percentage of the wild-type activity.

NDP Kinase Assay. NDP kinase activity was measured in a coupled enzyme assay with ATP as the phosphate donor and dTDP as the phosphate acceptor nucleotide. This assay measures the extent of ADP formation from ATP using pyruvate kinase and lactate dehydrogenase, as described previously (*20*). The average specific activity of the wild-type protein was 731 units/mg. The specific activities of the mutant proteins were calculated on the basis of several independent determinations and are expressed in Table 2 as a percentage of that of the wild-type enzyme.

Determination of the Oligomerization State of NM23-H2 Mutants. The molecular size of NM23-H2 wild-type and mutant proteins was determined by conventional size exclusion chromatography on a 27 cm × 1.5 cm Sephacryl S-200 HR (Pharmacia) column, as described previously (*21*). All proteins that were tested were found to be hexameric.

RESULTS

Strategy Used for Mutagenesis. This rationale for mutagenesis was based on the fact that Lys12, the critical amino acid in the DNA cleavage reaction, is located in the nucleotide-binding site along with other conserved residues implicated in the NDP kinase reaction (*1*−*5*). Thus, using the crystal structure of an NM23-H2−GDP complex (*3*), we targeted these amino acids for mutagenesis in the nucleotide-binding region. We sought to determine, for example, whether residues involved in nucleotide recognition, e.g., Tyr52, Tyr67, Arg88, Arg105, Asn115, and W149, and, in particular, His118, the phosphorylation site of the NDP kinase reaction, play a role in DNA cleavage. In addition, we targeted Pro101, which is known to be involved in stabilizing the trimeric structure (*23*), Ser120 because it has been found to be mutated in neuroblastoma (*24*), and the partially conserved Pro122 found mutated in melanoma (*2*). We also targeted Thr7, Pro13, Asp14, and Asn17, because of their proximity to Lys12, and because of their potential role in a glycosylase/lyase-like mechanism (*7*). For each mutation, several independently purified enzyme preparations were made and analyzed for covalent complex formation, DNA cleavage, and NDP kinase activities. All of our proteins were purifed at 0−4 °C and stored at −80 °C, and the cleavage assays were performed at 0 °C. Under these conditions, we have observed no loss of activity over a period of weeks or months for enzymes kept at 0 °C. A representative sample of each of the important mutants was tested for the state of oligomerization and was found to be hexameric.

Table 2: Functionally Important Amino Acid Residues of NM23-H2

DNA binding[a,b]			
sequence-specific	covalent	DNA cleavage[b]	NDP kinase[c]
R34	K12	K12	K12
N69	R88	R88	R88
K135	R105	R105	R105
		Q17	Y52
			N115
			H118

[a] From ref *21*. [b] From ref *7* and this paper. [c] From ref *20* and this paper.

Covalent DNA Binding by Mutants of NM23-H2. To determine whether any of the amino acids in the catalytic region, besides Lys12, can participate in covalent DNA binding, the mutants were examined for covalent complex formation using a heat-trapping assay (*7*). If a Schiff base intermediate is produced between DNA and Lys12, it can be trapped by heat treatment or by reduction with sodium borohydride, yielding a covalently linked enzyme−DNA complex which can be detected with denaturing SDS−PAGE gels. Out of 17 amino acids that were tested, two, besides Lys12, appeared to be critical for covalent DNA binding: Arg88 and Arg105 (Table 1 and Figure 2, the latter showing the results of a representative experiment). Because Arg88 and Arg105 were not found on the peptide bound to DNA covalently in the previous study (*7*), and because arginine, as demonstrated by the K12R protein, is capable of forming a Schiff base (*7*), we assume that Arg88 and Arg105 are required for the DNA cleavage reaction for other reasons, such as base recognition or provision of basic conditions that are necessary for catalysis (see the Discussion). The phenotype of the alanine-substituted mutant K12A confirms our previous finding that the ϵ-amino group of Lys12 is the functional group responsible for covalent DNA binding.

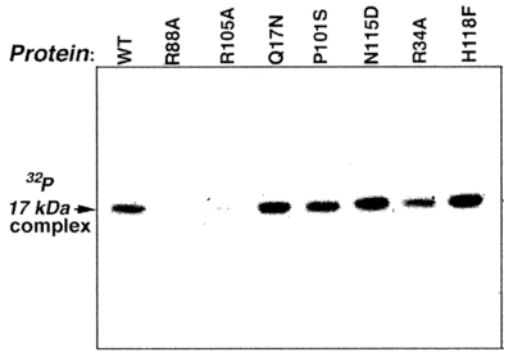

FIGURE 2: SDS−PAGE analysis of covalent protein−DNA complexes formed between plasmid DNA uniformly labeled with ^{32}P and site-directed mutants of NM23-H2. Shown is an autoradiogram of a dried gel. Positions of the ~17 kDa protein subunits radiolabeled with the covalently attached DNA are shown at the side. The concentrations of each of the proteins in the assays were equivalent. The figure shows results from a representative experiment.

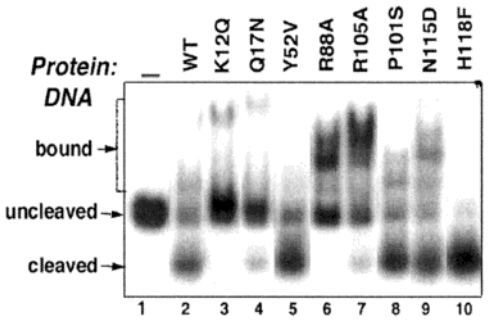

FIGURE 3: DNA cleavage activities of NM23-H2 point mutants by EMSA analysis. A 45 bp duplex oligonucleotide was incubated with equivalent amounts of each protein, and the samples were subjected to native gel electrophoresis as described in Experimental Procedures. Shown is an autoradiogram of an exposed gel from representative cleavage experiments. The positions of free, bound, and cleaved DNA substrates are indicated with arrows on the left. The lane 1 sample contained no protein.

DNA Cleavage by Mutants. To test mutants for DNA cleavage activities, a standard mobility shift assay (EMSA) was used in which both the slower-migrating protein−DNA complexes and the cleaved DNA products can be resolved in a single 5% native polyacrylamide gel. The results of these experiments are shown in Table 1, and representative cleavage data are depicted in Figure 3. Besides K12Q, three additional mutations (Q17N, R88A, and R105A) produced cleavage-defective proteins. The mutant K12A, like K12Q previously, was also inactive (while K12R had retained some cleavage activity), confirming that the ϵ-amino group of Lys12 is critical for the cleavage reaction (*7*). It is important to note here that despite its DNA cleavage defect, the Q17N mutant was not impaired in covalent DNA binding (Figure 2 and Table 1). The mutant proteins P13G and D14E had exhibited reduced cleavage activity, which may be significant because these residues are neighboring K12 and are conserved in most NM23 sequences. On the other hand, the Y52V, Y67F, and H118F mutants were absolutely normal, while the N115D protein demonstrated only a minor cleavage defect. The data in Figure 3 also illustrate that overall, the sequence-dependent, noncovalent DNA binding was variable among the mutants, and that some, e.g., R88A and R105A, exhibited even enhanced DNA binding. As appears in Figures 3−5, the protein-bound DNA complexes can have different

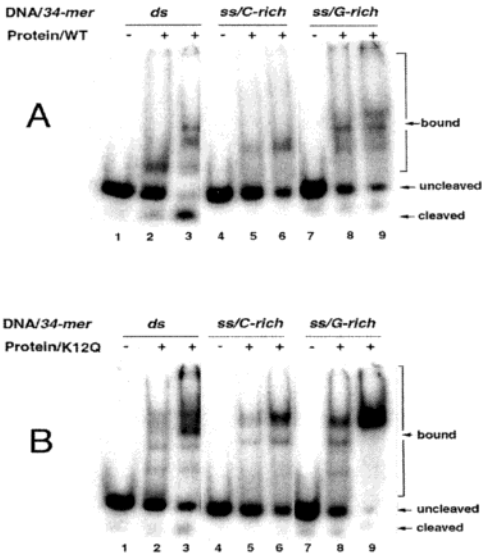

FIGURE 4: Single-stranded oligonucleotides are not substrates for DNA cleavage. (A) Autoradiogram showing the exposed gel from the cleavage experiment with a 34-mer duplex oligonucleotide (lanes 1−3) or the equvalent single-stranded C-rich top strand (lanes 4−6) and a G-rich bottom strand (lanes 7−9). Each substrate was incubated with equivalent amounts of wild-type protein and subjected to native gel electrophoresis as described in Experimental Procedures. (B) Same experiment as depicted in panel A with the cleavage-deficient K12Q mutant. Positions of the free, bound, and cleaved substrates are indicated with arrows on the left. Samples in lanes 1, 4, and 7 contained no proteins. Samples in adjacent lanes labeled with a + contain 250 and 500 ng of each protein, respectively.

mobilities. The fastest-migrating complex above the uncleaved DNA is probably protein bound to cleaved DNA, because this band does not appear in the absence of DNA cleavage. The other, slower-migrating bands are almost certainly complexes formed between one and four molecules of protein and uncleaved DNA fragments, because they can appear with both cleavage-defective and cleavage-proficient mutants. It should be pointed out that the substrate DNA sequences used here all contain three perfect directly repeated CCTCCC elements and one imperfect directly repeated CCTCCC element (see Experimental Procedures), which are recognition sites for the cleavage reaction (*22*). In prior experiments, the sequence-dependent DNA binding-deficient mutants R34A, N69H, and K135H failed to cleave DNA (*22*). The results of additional experiments shown in Figure 4 demonstrate that while both the wild-type NM23-H2 and the K12Q cleavage mutant can bind to single-stranded oligonucleotides, the preferred cleavage substrate of NM23-H2 is double-stranded DNA.

NDP Kinase Activity of Mutants. NDP kinase activity was assessed in a coupled kinetic assay using pyruvate kinase and lactate dehydrogenese that assess ADP formation from ATP, the phosphate donor nucleotide, with dTDP as the phosphate acceptor (*20*). The results summarized in Table 2 show that while some of the mutations had no or only a minor effect on catalysis, others significantly reduced the activity, and several of the mutants were completely inactive. Overall, in addition to residues H118 (*20*, *23*) and K12 (*7*), which were identified previously as being critical for the NDP kinase activity of NM23-H2, we show here that residues Y52, R88, R105, and N115 are also essential (Table 1). The equivalent residues have already been described as

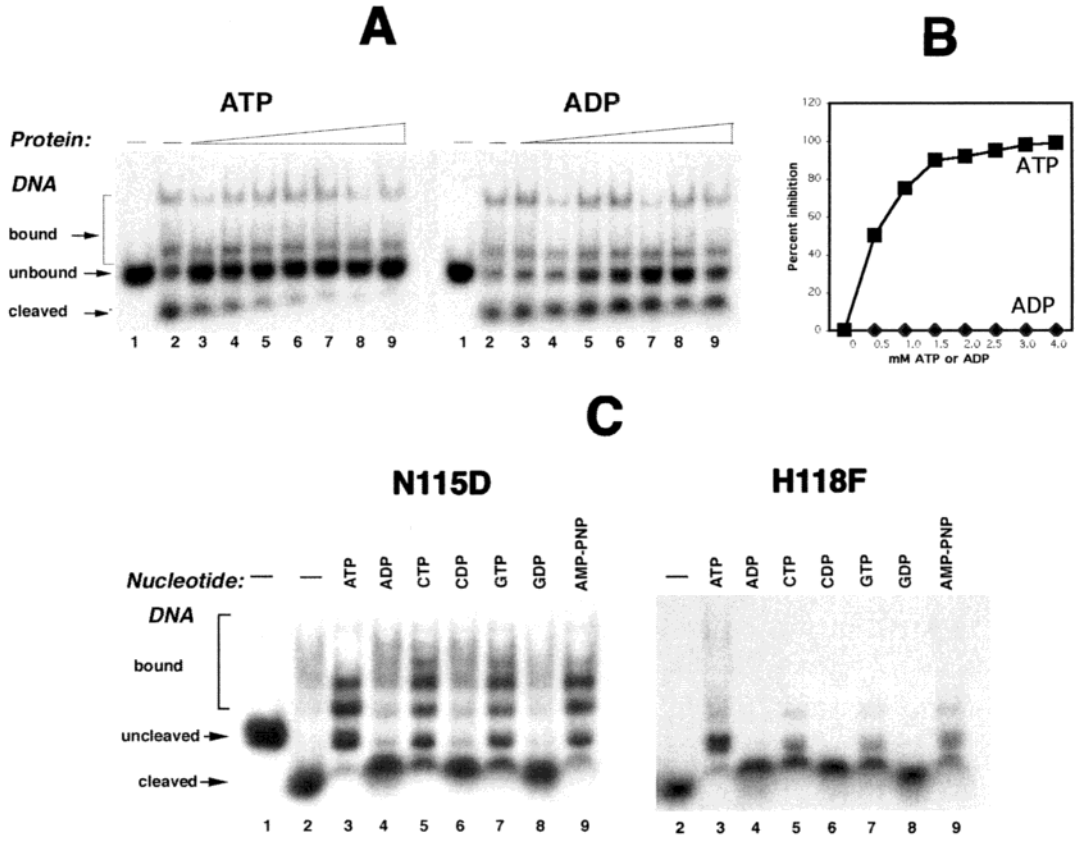

FIGURE 5: Nucleoside triphosphates inhibit the cleavage of DNA. (A) Autoradiogram showing an exposed gel from the cleavage experiment with a 45 bp duplex oligonucleotide incubated with the wild-type enzyme and increasing concentrations (from 0 to 4 mM) of ATP (left) and ADP (right). The positions of the free, bound, and cleaved substrates are indicated with arrows on the left. Lane 1 samples contained no protein. (B) Quantitation of the results of the experiment shown in panel A. (C) Cleavage experiment with the NDP kinase-deficient N115D (left) and H118F (right) mutants in the presence of each nucleotide (4 mM) as indicated in the top row. Lane 1 contains no protein. Lanes 2−9 contain equivalent amounts of each mutant.

being important for NDP kinase activity of the *Dictyostelium* enzyme (*26*).

Table 2 summarizes the important amino acids for sequence-dependent and covalent DNA binding, for DNA cleavage, and for the NDP kinase activities. Of the six amino acid residues established as being essential for the NDP kinase reaction, three of them (K12, R88, and R105) are also critical for the covalent DNA binding and cleavage reactions. Since the cleavage-defective mutant Q17N was not impaired in covalent DNA binding, this mutant, along with H118F, Y52V, and N115D, has in essence uncoupled the covalent DNA binding step from the cleavage and from the NDP kinase reactions. The NDP kinase catalytic residue His118 (and, by inference, the phosphotransferase/NDP kinase activity) is not required for any of the DNA-related functions examined here, nor is it essential for sequence-dependent DNA binding, or in vitro transcription as prior studies have shown (*20*). It is also worth noting that all of the amino acid residues found to be essential for the NDP kinase reaction have been conserved, while those required for the sequence-dependent DNA binding, and one out of four required for the cleavage reaction, are not. On the other hand, not all of the conserved amino acids appear to be relevant to either the nuclease or the NDP kinase activity of NM23-H2 as shown in Table 1 (e.g., Y67, S70, S120, and W149).

Nucleoside Triphosphates Differentially Inhibit DNA Cleavage by NM23-H2. Prior studies have shown that ATP inhibits DNA cleavage by NM23-H2 and *E. coli* NDP kinase, but does not inhibit sequence-dependent DNA binding (*22*, *25*). Here we examined the relationship between DNA cleavage by NM23-H2 and His118 phosphorylation, by testing a range of nucleotide substrates and analogues, first using the wild-type protein. In panels A (left panel) and B of Figure 5, increasing amounts of ATP included in the reactions inhibited DNA cleavage, with 50% inhibition obtained in the presence of 0.5 mM ATP. ADP, on the other hand, did not inhibit DNA cleavage at these concentrations [Figure 5A (right panel), Figure 5B, and Table 3]. As shown in panels A and C of Figure 5, these amounts of ATP and ADP did not affect the sequence-specific DNA complex formation.

Table 3: Inhibition of DNA Cleavage by Nucleotides

nucleotide	percent inhibition[a]	nucleotide	percent inhibition[a]
ATP	100	dATP	80 ± 8.9
AMP-PNP	95 ± 9.8	dCTP	80 ± 8.9
CTP	70 ± 8.4	dGTP	55 ± 7.4
GTP	75 ± 8.7	dTTP	70 ± 8.4
UTP	80 ± 8.9	dADP	10 ± 3.1
ADP	5 ± 2.2	dCDP	<1
CDP	5 ± 2.2	dGDP	<1
GDP	<1	dTDP	7.5 ± 2.7
UDP	5 ± 2.2		

[a] Percent inhibition of cleavage by 4 mM nucleotide of wild-type protein.

Several other ribo- and deoxyribonucleotide triphosphates also inhibited the cleavage reaction, albeit less effectively

than ATP (Table 3 and Figure 5C), while nucleoside diphosphates had none or only marginal effects (Table 3 and Figure 5A−C). Overall, ATP was the most potent inhibitor, with the nonhydrolyzable nucleotide analogue adenylylimidodiphosphate (AMP-PNP) nearly as effective, indicating that neither ATP hydrolysis nor His118 phosphorylation is responsible for the inhibition of DNA cleavage by triphosphates. Overall, the order of inhibition was as follows: ATP > CTP, GTP, and UTP > dATP, dCTP, dGTP, and dTTP (Table 3). Thus, the adenine base and the presence of a γ-phosphate are the most important aspects of this cleavage inhibition. Since sequence-dependent DNA−protein complex formation is not affected by the presence of nucleotides, and because ATP, but not ADP, inhibited covalent DNA binding (not shown), the inhibition by ATP must occur at the covalent DNA binding step.

However, since AMP-PNP is potentially able, albeit slowly, to phosphorylate His118, a more decisive test would be the inhibition by ATP of cleavage by the His118 protein. Thus, we tested two of the NDP kinase-deficient mutants for inhibition by nucleotides, H118F and N115D (Figure 5C), both of which are functional nucleases (Table 1 and Figure 3). As was the case for the wild-type protein, ATP and AMP-PNP also inhibited DNA cleavage by these mutants, confirming that His118 phosphorylation and NDP kinase activity are not essential for nucleotide inhibition.

DISCUSSION

NM23 proteins regulate a variety of cellular processes, but the underlying biochemical mechanisms are not known. It has been presumed for decades that the in vivo function of NM23/NDP kinases is in nucleic acid metabolism through the synthesis of nucleoside triphosphates (*1*, *4*, *5*, *14*, *23*). More recently, DNA structural changes, via a covalent DNA cleavage mechanism, have also been postulated as a mode of action (*7*, *22*, *25*). It has also been suggested that the nuclease and the NDP kinase share the nucleotide-binding site as their catalytic region (*7*). In this study, we conducted a series of experiments aimed at elucidating the role of active site amino acids in the enzymatic activities of NM23-H2, by replacing residues that, on the basis of the known crystal structure, can make contact with nucleotides (*3*), or, we supposed, might play a role otherwise. On the basis of functional assays, three different types of mutations were identified (Tables 1 and 2): (1) mutations that inactivated all three, covalent DNA binding, DNA cleavage, and NDP kinase activities (K12Q and -A, R88A, and R105A); (2) one mutation that blocked only the DNA cleavage reaction (Q17N); and (3) mutations that abolished only the NDP kinase activity (Y52V, N115D, and H118F). These findings clearly establish the presence of both shared and separation-of-function residues, and suggest that NM23-H2 may possess multiple biochemical activities. This is further suggested by the observation that nucleoside triphosphates are inhibitors of the covalent DNA binding and cleavage mechanism, independent of His118 phosphorylation by ATP, and of the NDP kinase activity.

None of the catalytic mutants lost specific DNA binding activity, which requires a different DNA binding surface and a different set of residues (*21*), and all maintained their correctly folded quaternary structures. Remarkably, six of the seven residues involved in these reactions (K12, Y52, R88, R105, N115, and H118) are fully conserved from bacteria to humans, indicating similar active sites and similar mechanisms, and underscoring the biological importance of these reactions. The one exception, Q17, is partially conserved (*1*).

Lys12, Arg88, and Arg105 Play a Role in Covalent DNA Binding and in the Cleavage of DNA. Crystal structures of NM23/NDP kinases complexed with nucleoside diphosphates indicate that the nucleotide-binding site is in a cleft located on the enzyme surface (reviewed in ref *4*). The residues making contact with the nucleoside diphosphates all have charged or polar side chains. In the NM23-H2−GDP crystal structure, Lys12, located near the mouth of the nucleotide-binding cleft, makes contact with the 2′- and 3′-hydroxyls of the ribose (*3*). Lys12 is also essential for the NDP kinase activity of NM23-H2 (ref *7* and this paper), and its equivalent, Lys16, is important for *Dictyostelium* NDP kinase (*26*). Because the crystal structure of the K16A mutant of *Dictyostelium* NDP kinase does not demonstrate any structural defects (*27*), mutation of Lys12 in NM23-H2 is unlikely to be causing the functional defects described in this paper.

Lys12 is the main catalytic residue of the DNA cleavage reaction. The role of Lys12 in DNA cleavage has been examined by replacement with glutamine (K12Q), alanine (K12A), and arginine (K12R), indicating that the reaction requires the ϵ-amino group (ref *7* and this paper). The ϵ-amino group of an active site lysine is also the critical functional group in the DNA cleavage reaction of the bifunctional DNA glycosylase/lyase BER enzymes (*28*). Were NM23-H2 to cleave DNA via a glycosylase/lyase-like mechanism, it would be expected to bind to C1 of the sugar, a binding mode that would be different from mononucleotide binding as indicated by the structural data (*3*).

Both Arg88 and Arg105 (Figure 2 and Tables 1 and 2) are also required for covalent DNA binding and cleavage catalysis. These two residues, together with Lys12, could well provide the second DNA-binding motif or a covalent DNA-binding pocket for NM23-H2. Arg88 and Arg105 could also play a structural role in maintaining the geometry of the active site, and may stabilize hydrogen bonding by the Schiff base precursor. Both of these residues make contact with the α- and β-phosphates of GDP in the crystal structure (*3*), and are essential for the NDP kinase activity of NM23-H2 (Table 1). The equivalent residues Arg92 and Arg109 are also essential for the NDP kinase activity of the *Dictyostelium* enzyme (*26*).

Role of Gln17. Mutation of Gln17 has resulted in the loss of DNA cleavage activity but not of covalent DNA binding (Figures 2 and 3 and Table 1), suggesting that these two steps are separable. Although the Q17N protein can form a noncovalent complex with DNA, this binding is not exaggerated, compared with other cleavage-deficient proteins K12Q, R88A, and R105A (Figures 3 and 4). It is therefore possible that mutation of Gln17 affects the sequence-specific DNA binding activity to some degree, because, like the other sequence-dependent DNA-binding residues, Gln17 also lies on the equator. On the other hand, Gln17 is unlike these residues in that it is conserved in at least half of the NDP kinases, whereas Asn69 and Lys134 are not, not even between the most closely related homologues NM23-H1 and

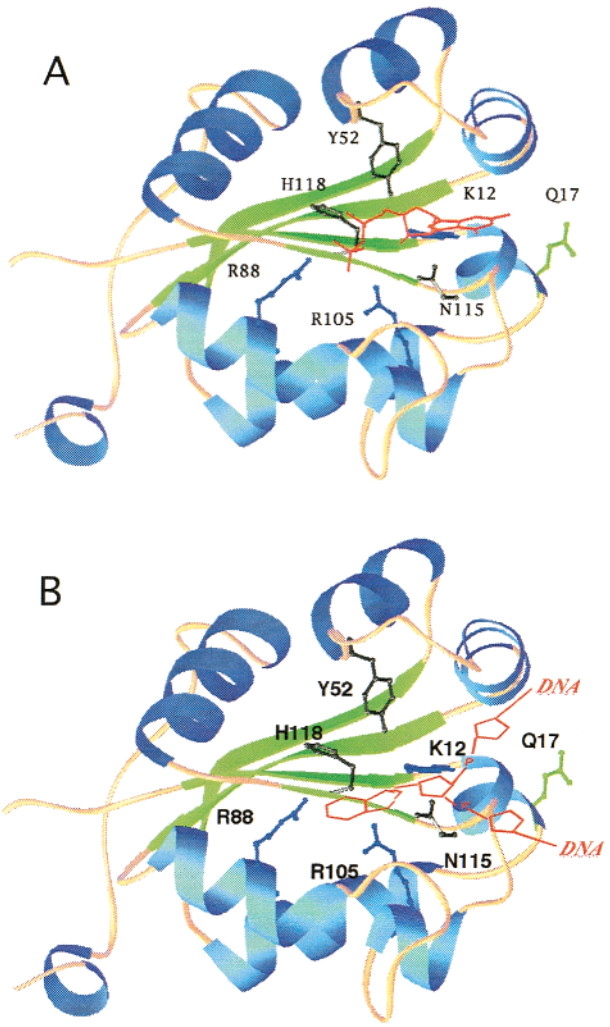

FIGURE 6: Ribbon diagram of an NM23-H2 monomer showing the nucleotide binding site and side chains of the catalytically important amino acid residues described in Tables 1 and 2. Side chains of residues shown in blue (K12, R88, and R105) are required for covalent DNA binding, DNA cleavage, and NDP kinase activities. Q17 (green) is required only for the DNA cleavage step. Y52, N115, and H118 (side chains in black) are required only for the NDP kinase activity. (A) The complex shown with GDP (red), with the two phosphate groups pointing toward the catalytic His118 residue (from ref *3*). (B) A hypothetical DNA mode of binding of NM23-H2 to duplex DNA based on the mutational and functional data, in which the covalent cleavage of duplex DNA by NM23-H2 occurs via a nucleotide flipping mechanism (*28*). Although the substrate used in these studies is double-stranded DNA, for the sake of simplicity only one DNA chain is shown with a flipped nucleotide (red). For more details, see the text. Note that K12, R88, and R105 would be in position to interact with the nucleoside. All of the residues shown are conserved and involved in binding of nucleotides, with the exception of Q17N, which is present in only half of the NDP kinase sequences. This model is different from the model proposed for single-stranded oligonucleotide binding to NM23-H2 by Raveh et al. (*31*), where the mode of binding of the terminal base to the active site is similar to the binding orientation of GDP in the complex depicted in panel A.

-H2. One might suppose that Gln17 (see Figure 6) has a role in contacting a base and securing DNA to the binding pocket, similar to the role of a Gln residue in the proximity of the catalytic lysine for DNA glycosylase/lyases (*29*). However, since Gln17 is not conserved in the *E. coli* sequence, this residue must not be needed for the otherwise conserved cleavage reaction by the *E. coli* enzyme. Moreover, Gln17 is not involved in the phosphate transfer reaction, because the Q17N mutant protein is a perfectly functional NDP kinase (Table 1).

Role of His118 and DNA Cleavage Inhibition by NTPs. His118 is buried in the active site and is the most important residue for the NDP kinase; it is involved in nucleotide binding and, during NDP kinase catalysis, plays the role of the phosphate donor and acceptor. However, His118 is not involved in any of the DNA-related functions examined here (covalent DNA binding and DNA cleavage), or previously (sequence-specific DNA binding and transcriptional activation in vitro; *20*). This may be significant in light of the absolute conservation of His118 and its proximity to Arg88 and Arg105, both of which are also conserved and are both required for the covalent DNA binding and cleavage reactions. And, consistent with the observation that His118 phosphorylation is not the cause of inhibition of covalent DNA binding and cleavage by NTPs, as demonstrated by two findings (1) NTPs also inhibited the cleavage activity of the H118F mutant, which cannot be phosphorylated by ATP (Figure 5C), and (2) AMP-PNP, a nonhydrolyzable ATP analogue, can inhibit the DNA cleavage reaction nearly as effectively as ATP.

One explanation for the NTP inhibition may be geometric; i.e., NTPs may occupy sufficient space to occlude the active site and to prevent the binding of a DNA nucleoside. Alternatively, the presence of an NTP may structurally perturb the binding site such that it can no longer accommodate a segment of a duplex oligonucleotide or a nucleotide flipped out of a DNA helix. Nucleoside diphosphates, on the other hand, may not interfere with DNA binding because, by lacking the γ-posphate, they occupy less space and may not disrupt the active site structure. Clearly, NTP binding per se, not NTP hydrolysis, is responsible for this DNA cleavage inhibition. While inhibition is most effective with ATP, beyond that, there is little difference between the various nucleotides with respect to either the base or the ribose hydroxyls. Because ATP is the most abundant nucleoside triphosphate in the cell, and because it seems to be the most potent inhibitor of DNA cleavage, it is possible that in vivo ATP regulates the cleavage activity through His118 phosphorylation and/or dephosphorylation. His118 may also play a role in a DNA repair mechanism, by regulating the supply of dNTPs to sites and/or gaps of cleavage or, perhaps, by eliminating mutagenic precursors from the nucleotide pool by a phosphorylation and/or dephosphorylation mechanism.

Mononucleotides and DNA Bind Differently in the Catalytic Site of NM23-H2. In the NM23-H2−GDP crystal structure, each of the six nucleotide-binding sites of the hexameric protein is in complex with a GDP molecule. The guanine base (Figure 6A) is largely on the enzyme surface, where it makes only nonspecific polar contacts. The ribose is totally buried in the protein, with the 2′- and 3′-OH groups hydrogen bonding to the Lys12 and Asn115 side chains. The phosphates are also buried, pointing toward the active site His118, and interacting with the guanidinium group of Arg88 (*3*). The mode of binding of GDP to NM23-H2 is essentially the same as that of other nucleotide substrates in complex with NDP kinase in other known X-ray structures (reviewed in ref *4*).

There is no direct explanation for how DNA binds to the nucleotide-binding site in NM23-H2 on the basis of the experimental data presented here. However, we do know from existing structural data that the nucleotide-binding cleft is not large enough (20 Å long, 6 Å wide, and 10 Å deep) to accommodate a double-stranded DNA oligonucleotide, unless the protein undergoes significant structural changes in the process (*3*). In the absence of conformational changes, one mode of DNA binding in the nucleotide-binding site might be extrahelical, where a nucleotide is flipped out of the double helix into the active site (Figure 6B). Such a nucleotide flipping mechanism is an obligatory step in the DNA cleaving reaction of DNA glycosylase/lyases (reviewed in ref *28*), and would be consistent with the Lys12-based cleavage chemistry of NM23-H2. In the glycosylase/lyase reaction, lysine acts both to remove a base and to cleave the DNA strand. Depending on the substrate and the presence of ATP, the NM23-H2 enzyme could flip-flop between two different binding modes, where the binding orientation of the mononucleotides is opposite from the binding orientation of nucleosides from DNA (Figure 6A,B). A different model, based on UV cross-linking of a single-stranded oligonucleotide to NM23-H2, was proposed by Raveh et al. (*31*), where the terminal base of the aptamer binds inside the active site, similar to that of guanine in the GDP structure (Figure 6A). The functional relevance of single-stranded oligonucleotide binding by NM23-H2, however, remains to be elucidated.

NM23-H2 Is a Multifunctional Protein. NM23-H2 is a unique and hitherto unknown type of enzyme that seems to be involved in several complex biochemical mechanisms that are probably related. The various biochemical activities documented thus far for NM23-H2/PuF/NDPK-B include nucleotide binding (six nucleotides per hexameric molecule; reviewed in ref *4*), nucleoside triphosphate synthesis (reviewed in ref *1*), and several activities related to gene regulation, including sequence-dependent DNA binding (*17*), covalent DNA binding and reversible DNA cleavage (*22*), transcriptional regulation (*13*, *17*, *18*, *30*), and plasmid recombination (*22*). Additionally, there have been reports from other laboratories that NDP kinases possess a protein phosphotransferase activity in vitro; however, recent experiments demonstrate that these results were probably artifactual and that NDP kinase does not behave as a true protein kinase (*25*).

Because of the remarkable conservation of the catalytic site (*1*), and the conservation of the NDP kinase and DNA cleaving activities from humans to *E. coli* (*25*), it is rather unlikely that either of these activities is merely incidental. It would also be extraordinary to have this many conserved residues so entangled within a single nucleotide-binding site (Figure 6), and yet serving vastly different functions. In the end, it is more likely that the NDP kinase and the DNA cleavage activities are part of a single mechanism, and that a combination of these may be essential for protein function in the cell.

ACKNOWLEDGMENT

We thank J. Stock for helpful comments on the manuscript, N. Gordunov for technical assistance, and S. Bonette for secretarial help.

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BI025606+