Magnesium and Phosphate Ions Enable NAD Binding to Methylenetetrahydrofolate Dehydrogenase-Methylenetetrahydrofolate Cycohydrolase*

Karen E. Christensen1, I. Ahmad Mirza1,2, Albert M. Berghuis4,5, and Robert E. MacKenzie6,7

From the 1Department of Biochemistry and the 6Department of Microbiology and Immunology, McGill University, Montréal, Québec H3G 1Y6, Canada

The mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase (NMDMC) is believed to have evolved from a trifunctional NADP-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase-synthetase. It is unique in its absolute requirement for inorganic phosphate and magnesium ions to support dehydrogenase activity. To enable us to investigate the roles of these ions, a homology model of human NMDMC was constructed based on the structures of three homologous proteins. The model supports the hypothesis that the absolutely required Pi can bind in close proximity to the 2'-hydroxyl of NAD through interactions with Arg166 and Arg198. The characterization of mutants of Arg166, Asp190, and Arg198 show that Arg166 is primarily responsible for Pi binding, while Arg198 plays a secondary role, assisting in binding and properly orienting the ion in the cofactor binding site. Asp190 helps to properly position Arg166. Mutants of Asp190 suggest that the magnesium ion interacts with both Pi and the aspartate side chain and plays a role in positioning Pi and NAD. NMDMC uses Pi and magnesium to adapt an NAD binding site for NAD binding. This adaptation represents a novel variation of the classic Rossmann fold.

During embryogenesis and tumorigenesis mammalian mitochondria use a folate-dependent pathway to generate both glycine and one-carbon units to support cytoplasmic purine synthesis (1, 2). One of the enzymes in this pathway, the NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase (NMDMC),5 catalyzes the interconversion of 5,10-methenyltetrahydrofolate (methylene-THF) and 10-formyltetrahydrofolate (formyl-THF) in mammalian mitochondria. The mitochondrial formyl-THF is converted to formate by a monofunctional formyl-THF synthetase and is released to the cytoplasm for re-conversion into formyl-THF to support purine biosynthesis (1). NMDMC can use both NAD and NADP as a cofactor in its dehydrogenase activity, although the maximal activity with NADP is only about twenty percent of that with NAD (3). NMDMC is thought to have evolved from a trifunctional NADP-dependent methylene-THF dehydrogenase-methenyl-THF cyclohydrolase-formyl-THF synthetase (DCS) through the loss of the synthetase domain and the change in cofactor specificity from NADP to NAD (4). This change in cofactor specificity is important because the use of NAD rather than NADP in mitochondria shifts the equilibrium of the reaction to favor the production of formyl-THF (5). The increased production of formyl-THF is required to meet the demands for glycine and purines during embryogenesis (1, 2, 6). However, it is not known how this cofactor specificity change was accomplished.

NMDMC is unique in its absolute requirement for magnesium and inorganic phosphate ions for NAD-dependent dehydrogenase activity and magnesium ions for NADP-dependent dehydrogenase activity (3, 7). However, neither ion is essential for the cyclohydrolase activity. The role of these ions in the dehydrogenase activity is not clear. The sequence of binding of the cofactors and substrates to NMDMC, as established kinetically by Yang and Mackenzie (3) and Rios-Orlandi and MacKenzie (7), suggests a role for Pi and Mg2+ in the binding of the cofactor; the ions bind to the protein first, followed by NAD and then the folate substrate. A preferred order of binding of the ions was not established; either ion appears to be able to bind to the enzyme and affect the binding of the other. These results suggested a possible interaction between the two ions in the binding site.

The observation that Pi competitively inhibits the cofactor in NADP-dependent dehydrogenase assays of NMDMC led to the proposal that Pi may occupy a position adjacent to the 2'-hydroxyl of NAD, close to the space that would be occupied by the 2'-phosphate of NADP (3). Previous work on the DC domain of the human NADP-dependent DCS identified two residues (Arg173 and Ser197) as being important to the binding of NADP to the enzyme through its 2'-phosphate (8). Sequence alignments of mitochondrial NAD-DCs with trifunctional NADP-DCs suggest that Arg166 and Arg198 (numbered from the amino-terminal glutamate of the mature enzyme) may interact with Pi (9 and Fig. 1).

The crystal structure of the DC domain of the human NADP-dependent DCS has been determined both with bound NADP and with bound NAD and folate analogues (10, 11). The structure of the Escherichia coli NADP-dependent DC has been determined by x-ray crystallography in the absence of bound substrates (12), and the structure of the Saccharomyces cerevisiae NAD-dependent dehydrogenase has been determined with and without bound NAD (13). However, since no crystal structure of NMDMC has yet been obtained, we constructed a homology model of the enzyme based on
three related structures and used this to locate the P$_i$ and Mg$^{2+}$ binding sites using site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

*Materials*—(R, S)-tetrahydrofolate was synthesized according to the method of Drury *et al.* (14) and stored in sealed glass vials at 4 °C. Nickel-nitrilotriacetic acid-agarose was obtained from Qiagen. Vent DNA polymerase was obtained from New England Biolabs, and restriction enzymes were products of New England Biolabs and MBI Fermentas. Oligonucleotide primers were obtained from Sigma Genosys, Qiagen Operon, and Integrated DNA Technologies, Inc. NAD, imidazole, and rifampicin were purchased from Sigma. (R, S)-methenyltetrahydrofolate was obtained from B. Schircks Laboratories (Jona, Switzerland). All other chemicals and reagents were purchased from Bioshop or BDH and were of analytical grade.

*Construction of the Homology Model of Human NMDMC*—Crystal structures for three NMDMC homologues have been determined. The DC domain of the human NADP-dependent DCS crystallized with NADP and a folate analogue (PDB ID: 1DIB), the *E. coli* NADP-dependent DC (PDB ID: 1B0A), and the *S. cerevisiae* NAD-dependent dehydrogenase crystallized with NAD (PDB ID: 1EE9). A. alignment of the primary structures of NMDMC and the templates adjusted to reflect the fit of the crystal structures 1B0A and 1EE9 to 1DIB. Residues that are aligned in the sequence are considered structurally homologous. α-Helices are marked in red and β-sheets in blue. Residues targeted in mutagenesis experiments are marked with an asterisk. B and C, overlay of the NMDMC model and the template structures, showing one monomer of the dimer from two sides. The 1B0A structure is cyan, the 1DIB structure is magenta, the 1EE9 structure is blue, and the NMDMC model is green.
percent identity ranges between 22–43%, the three-dimensional structures are highly homologous (see Fig. 1).

A multiple sequence alignment of the target and template sequences was generated using ClustalW (15). The alignment was then refined to include structural data using alignments to the 1DIB structure generated using a brute force structural alignment method followed by iterative cycles of improvement as implemented in the program Lsqman (16). Structural elements for the model were selected from the templates by highest sequence identity to NMDMC. The structural elements from the aligned templates were linked together and the side chains mutated using the computer program O (17) with the Lovell et al. (18) rotamer data base.

The loop from residue 182 to 190, unique to mitochondrial NAD-dependent DCs (9 and Fig. 1), lacks structural data and was not included in the final structure. The position of Asp190 suggested that it could play a role in the cofactor binding site, so it was included in the model, and its position was determined by local energy minimization using CNS (19). The loop from residue 244 to 252 is disordered in the 1DIB structure and is not present in the E. coli and S. cerevisiae enzymes. This loop has been shown not to be required for DH activity or cofactor binding (20) and so was not included in the model. The geometry and stereochemistry of the model structure was optimized using CNS (19). For the optimization residues 243 and 253 were fixed to keep their position homologous to the 1DIB structure. NAD, P, and NADP were docked into the model based on the position of the cofactors in the template structures. The crystal structures 1B0A and 1EE9 show only one monomer of the enzymes and so the resulting model showed only one monomer of NMDMC, which is known to be a dimer (21). To model the dimer two copies of the model were superimposed on the 1DIB structure using Lsqman (16), and the geometry and stereochemistry of the side chains along the dimer interface was optimized using CNS (19). The quality of the model was evaluated at each step using Procheck (22). Pictures of the model were generated using Pymol (23).

Addition of Six-histidine Tags—To simplify purification a COOH-terminal six-histidine tag was added to NMDMC by PCR using primers including the 3’ terminus of the required coding region of the cDNA, the sequence of the tag and an XhoI site. The histidine-tagged cDNA was then subcloned into pBKeHB1 (24) to make pBKeHB1 303H6. The six-histidine tag replaces an unstructured tail made up of twelve residues that have no influence on enzyme activity. The kinetic constants and activities of 303H6 are identical to the full-length, non-histidine-tagged NMDMC (data not shown), and so the histidine tag does not interfere with the enzyme function. The truncated version of the protein was selected for this study because it had better expression levels than full-length NMDMC with six histidines added and to circumvent any problems that may arise from having the six-histidine tag added to the mobile unstructured tail. For simplicity, in the results section 303H6 is referred to as wild type, meaning unmutated pBKeHB1 303H6.

Site-directed Mutagenesis—Mutations were introduced into pBKeHB1 303H6 using in vitro overlap extension PCR as in Sundararajan and MacKenzie (20). The entire insert of the resulting vector was sequenced by automated sequencing (Genome Québec) to confirm the integrity of each mutant.

All of the mutants in this study should affect the NAD(P) cofactor binding and thus only the dehydrogenase activity, leaving the cyclohydrolase activity and folate substrate binding kinetic constants as controls for gross disruptions of the protein structure. All of the mutants reported in this study retained significant cyclohydrolase activity. There were no significant differences between the \( K_m \) values for methylene-

| Enzyme | Specific activity (% WT) | NAD-DH | NADP-DH |
|--------|--------------------------|--------|---------|
| WT     | 22.5 ± 1.1 (100)         | 2.92 ± 0.06 (100) |
| R166A  | ND*                     | ND     | ND      |
| R166S  | ND                      | ND     | ND      |
| R166K  | ND                      | ND     | ND      |

* ND = not detectable (<0.01 \( \mu \text{mol min}^{-1} \text{mg}^{-1} \)).
THF, with either NAD or NADP as the cofactor, or for methenyl-THF for any of the mutants. This shows that there are no gross disruptions of the protein structure in the mutants and that the folate substrate binding site is unaffected by these mutations.

Protein Expression and Purification—pBKeHB1 303H6 constructs expressing histidine-tagged NMDMC were transformed into E. coli BL21 DE3. Overnight cultures of transformed bacteria were used to inoculate 100 ml of Terrific Broth supplemented with 200 μg/ml ampicillin and incubated at 37 °C with shaking at 250 rpm. Expression was induced when cultures reached an A600 1.0 to 1.2 by adding isopropyl 1-thio-β-d-galactopyranoside to a final concentration of 2 mM. After 30 min 15 μg/ml rifampicin was added to inhibit host translation. Cells were harvested after an additional 90 min by centrifugation for 20 min at 4000 rpm at 4 °C in a Sorvall RC-3. Pellets of ∼0.3 to 0.4 g cells were stored at −85 °C.

Frozen pellets were thawed on ice and resuspended in 10 ml of sonication buffer (0.1 M potassium phosphate (pH 7.3), 35 mM β-mercaptoethanol, 1 mM benzenzamide hydrochloride, and 1 mM phenylmethylsulfonyl fluoride). Resuspended cells were disrupted by sonication on ice using 10–12 pulses of 10 s each separated by intervals of 1 min. Lysates were cleared by centrifugation at 12,500 rpm at 4 °C in a Sorvall SS-34 rotor for 20 min. Proteam sulfate (0.1 volume of 10 mg/ml solution) was added to the supernatant, followed by an additional 20-min centrifugation at 12,500 rpm.

The proteam sulfate-treated crude extract was adjusted to contain 0.5 M NaCl and 15 mM imidazole and added to 10 ml of a 50% slurry of Ni-NTA-agarose resin in binding buffer (0.1 M potassium phosphate (pH 7.8), 0.5 M NaCl, 15 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 mM β-mercaptoethanol, and 20% (v/v) glycerol). The slurry was mixed for 1.5 h at 4 °C on a rotator, and the resin was collected by centrifuging at 1,000 rpm for 10 min in a Sorvall RC-3. The resin was then resuspended in 5 ml of binding buffer and packed into a 1.5-cm diameter column. The column was washed once at 0.25 ml/min with 3 volumes of binding buffer, followed by 3 volumes of binding buffer containing 50 mM imidazole. The enzyme was eluted at 0.1 ml/min with binding buffer containing 250 mM imidazole. Fractions containing the enzyme were identified by Bradford assay (25). All enzyme preparations were evaluated for purity by SDS-PAGE on 10% polyacrylamide gels. Protein concentration was determined by Bradford assay (25). All enzyme preparations were identified by Bradford assay (25).

Enzyme Assays and Kinetics—Dehydrogenase assays were performed after Yang and MacKenzie (3) and Pawelek and MacKenzie (26). Standard conditions buffer contained 25 mM MOPS (pH 7.3), 5 mM potassium phosphate (pH 7.3), 5 mM magnesium chloride, 2.5 mM formaldehyde, 0.2 mM (6R,S)-tetrahydrofolate, 36 mM β-mercaptoethanol, and 0.6 mM NAD. For NADP-dependent DH assays the assay mixture was modified by removing the potassium phosphate and NAD and including 2 mM NADP. Standard activity assays are reported as the average of three separate determinations performed in triplicate using a single fixed time point. The determination of kinetic constants was performed on mutants with sufficient activity using a multiple time point assay as in Schmidt et al. (11), typically using four time points at 2-min intervals and at least five variable substrate concentrations.

Cyclohydrolase assays were performed as described in Pawelek and MacKenzie (26), with 5 mM magnesium chloride added to the buffer. Results reported are the average of three separate determinations done in triplicate. The determination of kinetic constants was done as for the standard cyclohydrolase assay, using at least five methenyl-THF concentrations.

Enzyme assays lead Yang and MacKenzie (3) to propose that P1 binds to NMDMC in a position analogous to the location of the 2′-phosphate of NADP in DCS. The NAD binding site of the NMDMC model is shown

| Enzyme | NAD-DH | NADP-DH |
|--------|--------|---------|
| WT     | 22.5 ± 1.1 (100) | 2.92 ± 0.06 (100) |
| R198A  | 0.170 ± 0.032 (0.8) | 0.156 ± 0.021 (5.3) |
| R198S  | 0.207 ± 0.018 (0.9) | 1.54 ± 0.09 (53) |
| R198K  | 12.6 ± 1.2 (56) | 1.24 ± 0.05 (42) |

### RESULTS

**NMDMC Homology Model**

The homology model of NMDMC is in good agreement with the template structures. The root mean square deviation of the α carbons between the model and each template is as follows: 1D1B structure (human DCS), 1.23 Å; 1B0A structure (E. coli DC), 0.92 Å; and 1EE9 structure (S. cerevisiae D), 1.40 Å (see Fig. 1). The Ramachandran plot of the model demonstrates that 82.6% of residues are in core regions, 14.6% in allowed regions, and 2.8% in generously allowed regions. No residues are found in disallowed conformations. A ribbon diagram of the homology model, shown with docked P1 and NAD, is shown in Fig. 2. The coordinates for the model have been deposited in the Protein Data Bank with accession number 1ZN4.

**The Phosphate Binding Site of NMDMC**

The competitive inhibition of P1 against NADP in dehydrogenase assays lead Yang and MacKenzie (3) to propose that P1 binds to NMDMC in a position analogous to the location of the 2′-phosphate of NADP in DCS. The NAD binding site of the NMDMC model is shown...
compared with the NADP binding site of DCS in Fig. 3. The P1 modeled in the NMDMC structure is slightly displaced from NAD into a positively charged cavity made up of Arg173 and Ser197, respectively, of the human DCS, which are involved in NADP binding (8).

Arg173 of DCS forms multiple hydrogen bonds with and stabilizes the charge of the 2′-phosphate of NADP (10). When this residue is mutated, dehydrogenase activity of DCS is reduced to less than 2.5% of wild-type activity, and values of $K_m$ for NADP are greatly increased, showing the importance of this residue in binding the cofactor (8). In the homology model (Fig. 3) Arg166 appears to have the potential to form multiple hydrogen bonding interactions with P1 and can also contribute to stabilize the charge of P1 similar to the role of Arg173 in DCS. Therefore this residue was targeted for mutagenesis.

Ser197 of the human DCS forms one hydrogen bond with the 2′-phosphate of NADP (10) and was shown by mutagenesis to play a supporting role in cofactor binding (8). In NMDMC (Fig. 3) Arg198 is the homologous residue to Ser197 of DCS. In the homology model of NMDMC it appears that this residue has the potential to form hydrogen bonds with P1 and can also contribute to charge stabilization and so it too was targeted for mutagenesis.

Upon examination of the model, the side chain of Asp133, a residue unique to NMDMC, was observed behind Arg173 in a position that suggests an electrostatic interaction (Fig. 3). Mutation of this residue provides the opportunity to manipulate the position of Arg173 in the protein and observe the effects, without actually changing the nature of the side chain.

**Mutagenesis of Arg173**—Even conservative mutations of Arg173 abrogate dehydrogenase activity (TABLE ONE). This is consistent with the role of Arg173 in DCS. Arg173 is primarily responsible for NADP binding through the 2′-phosphate, which suggests that Arg166 has a similar role in binding P1 in NMDMC.

**Mutagenesis of Asp133**—To examine the effects of changing the position of Arg173 without altering the side chain Asp133 was mutated. The assay results are shown in TABLES TWO and THREE. Mutations of this residue that disrupted the ionic interaction of Asp133 and Arg173 (such as D190A and D190S) or resulted in a shift of the position of Arg166 in the binding site (D190E) resulted in the loss of DH activity. Only D190N retained significant amounts of DH activity with both cofactors. This mutant would have a weaker interaction between residues 166 and 190, allowing greater movement of Arg166, but the position of Arg166 would not shift due to the similar size of the Asp and Asn side chains. The affinity of D190N for P1, as measured by its inhibition constant against NADP, is not changed although all values of $K_m$ for substrates and ions are elevated. These changes in the $K_m$ values most likely reflect the inhibition of DH activity due to the difficulty of positioning the ligands properly in the binding site when Arg166, and therefore P1, are free to move. The increase in the Mg$^{2+}$ constants suggests that the Mg$^{2+}$ and P1 binding sites, like the NAD and P1 binding sites, interact with each other. The $K_m$ for NAD is less affected than that for NADP, because the 2′-phosphate is covalently attached and requires positioning of a single ligand rather than two separate molecules. Binding NADP might help to partially reposition Arg166 in the D190N mutant. These mutations show that the role of Asp133 is to position Arg166 in the binding site.

**Mutagenesis of Arg173**—The properties of Arg173 mutants are shown in TABLES FOUR and FIVE. In the case of R198A, where the arginine is substituted with a residue that is not capable of forming hydrogen bonds, both NAD-DH and NADP-DH activities are significantly reduced, indicating an important role for this residue (TABLE FOUR). When this arginine is substituted by serine, which is the homologous residue in DCS, NAD-DH activity is drastically reduced, while the NADP-DH is relatively unaffected. The $P_i$ affinity, as measured by the $K_i$ for $P_i$, is reduced in this mutant, which shows that the binding site of Mg$^{2+}$ is not distorted in R198S and that the combination of Arg166 and Mg$^{2+}$ is sufficient to allow wild-type affinity for NADP.

The R198K mutant, in which the arginine is replaced with a smaller and more flexible residue that also has a positive charge, retains roughly 50% of both NAD and NADP-dependent DH activities. In R198K all of the $K_m$ values are significantly increased compared with wild type. The $K_m$ values in this case likely reflect both the loss of affinity for $P_i$ (shown by the larger $K_i$ value for $P_i$), and the difficulty of properly positioning the $P_i$ in the binding site due to the increased flexibility of the side chain. The Mg$^{2+}$ constants, as in D190N, are also affected by this mutation, again showing that the two binding sites interact with each other. In this case, in contrast to R198S, the bulky side chain may be occluding the Mg$^{2+}$ site or distorting it in a way that impairs binding.

The role of Arg198 is illustrated by the effects of mutations on the $K_i$ value for $P_i$. Although R198S and R198K have roughly the same affinity for $P_i$ as measured by the $K_i$, R198S almost totally lacks NAD-DH activity, whereas R198K retains greater than 50%. Simply binding the phosphate is insufficient suggesting it must also be positioned properly for DH activity by an electrostatic interaction with a positive charge.
positions the phosphate that interacts with Arg^{166} and orients it within the binding site to interact with NAD.

The Magnesium Binding Site

Kinetics with Magnesium—A more sensitive multiple time point assay was used to determine $K_m$ values for this study, as opposed to the single time point assay used previously (3). This method revealed a previously unobserved co-operativity in the binding of Mg$^{2+}$ with NAD. The velocity versus [Mg$^{2+}$] curve with NAD as a cofactor is sigmoidal and fits to the Hill equation. The value of $n$ for 303H6 was found to be $1.99 \pm 0.10$ indicating near perfect co-operativity of binding. This effect is not observed in velocity versus [Mg$^{2+}$] curves using NADP as a cofactor nor when [P] is the variable substrate. Consequently both $K'$ and $n$ values are reported for magnesium. The significance of this apparent co-operativity is not clear.

Identification of Potential Magnesium Binding Sites—Potential Mg$^{2+}$ binding sites were identified by examining a multiple sequence alignment of methylene-THF dehydrogenases to find aspartate and glutamate residues that are conserved in human, mouse, and fruit fly NMDMC but not conserved in NADP-dependent enzymes that do not require Mg$^{2+}$. These residues in particular were selected because they are the preferred interacting partners of Mg$^{2+}$, at least one of them being found in all known Mg$^{2+}$ binding sites (27). This search narrowed down the potential interacting residues to 8 from 36. The local environment of these residues was examined in the homology model to determine whether any could contribute to a Mg$^{2+}$ binding site. The existence of a possible binding partner to permit charge balance, the size of the potential site ($\sim 5-6$ Å distance between binding partners), the charge of the site, and its access to the solvent were evaluated. Of the eight residues, only four are situated such that they could be part of a Mg$^{2+}$ binding site. Preliminary mutagenesis experiments eliminated three of these residues leaving Asp$^{133}$ as the only residue likely to contribute to a Mg$^{2+}$ binding site.

Mutagenesis of Asp$^{133}$—The properties of the Asp$^{133}$ mutants are shown in TABLES SIX and SEVEN. Substitution of aspartate by glutamate completely inactivates the DH activity with both NAD and NADP cofactors, indicating that the position of the carboxyl group is critical. However, D133A, D133S, and D133N, without free carboxyl groups, retain some DH activity. The D133S and D133N mutants show no reduction in affinity for P$_i$, as indicated by the $K_i$ values, but the values of $K_m$ for Mg$^{2+}$ are greatly elevated. These results support a role for Asp$^{133}$ in helping to bind Mg$^{2+}$. The loss of Mg$^{2+}$ affinity in these mutants affects the positioning of P$_i$ in the binding site and results in elevated kinetic constants for all the ligands. This suggests that the role of the Mg$^{2+}$ ion is to assist in the binding and positioning of P$_i$ much like the role of Arg$^{198}$.

DISCUSSION

The Position of Arg$^{166}$ Is Critical for Phosphate Binding—The model of NMDMC shows that residues Arg$^{166}$ and Arg$^{198}$ are homologous to Arg$^{173}$ and Ser$^{177}$ that interact with the 2’-phosphate of NADP bound to the human DCS. In NMDMC, Arg$^{166}$ and Arg$^{198}$ provide a positively charged pocket for binding P$_i$. Arg$^{166}$ is the residue that is primarily responsible for binding P$_i$; all mutants of this residue, even the conservative mutation to lysine, lack dehydrogenase activity. The lack of activity of R166K is not that surprising given that arginine residues are often favored over lysine at P$_i$ sites because they can form multiple interactions with the ion and can be resonance stabilized (28, 29).

Mutagenesis of Asp$^{190}$ shows that this residue is required to properly position Arg$^{166}$ in the binding site. This type of arginine-aspartate interaction to position the side chain has been previously observed in other proteins that bind phosphate ions (30, 31, 32). Mutation of Asp$^{190}$ to the larger glutamate residue displaces Arg$^{166}$ and essentially inactivates NMDMC. The D190N mutation retains significant DH activity. However, the weakened interaction with Arg$^{166}$ allows the residue to move, impeding ligand binding, which increases the ligand $K_m$ constants. These mutations show that the position of Arg$^{198}$ is critical for cofactor binding to NMDMC.

FIGURE 4. The magnesium ion binding site may be located in a cavity bounded by Asp$^{133}$, the inorganic phosphate, and the NAD cofactor, which could provide coordination points for the cation.
Magnesium and Phosphate Binding Sites of NMDMC

FIGURE 5. Comparison of the cofactor binding sites of alcohol dehydrogenase (PDB ID: 1HDX) (A), NMDMC (B), and the DC domain of the human DCS (C) (PDB ID: 1DBB). The diphosphate binding domain of NMDMC lacks the close contact of protein and cofactor that is seen in the classic NAD binding site of alcohol dehydrogenase. This domain is more similar to the DC domain of DCS, which is entirely dependent on interactions with the 2'-phosphate of NADP for cofactor binding (8). The magnesium and inorganic phosphate ions in the cofactor binding site of NMDMC set up a web of ionic and hydrogen bonding interactions that compensate for the lack of the covalent bond with the phosphate. This allows the adaptation of an NADP binding site to bind NAD.

ribose moiety is longer than the bond length of the 2'-phosphate of NADP. Therefore, when bound to the P₁ site of NMDMC, NADP will shift upwards in the binding site, moving the nicotinamide moiety slightly out of position, affecting the activity of the enzyme but not the affinity for NADP.

The Magnesium and Phosphate Ions Interface—Mutagenesis of the residues of the P₁ binding site supports the interaction of the P₁ and Mg²⁺ binding sites suggested earlier by the enzyme kinetics (3). Mutations such as D190N and R198K that alter the positioning of the P₁ in the binding site also result in reduced affinity for Mg²⁺ and indicate an interaction of the binding sites. The R198S mutant, which has decreased P₁ affinity (elevated Kᵦ) without altering NADP binding, has no effect on Mg²⁺ affinity. These results suggest that the phosphate ion itself might make up part of the Mg²⁺ binding site and suggests that the role of the Mg²⁺ ion is to assist in the binding and positioning of P₁, much like the role of Arg₁⁰⁸.

A Mg²⁺ ion interacting with Asp¹³³ could co-ordinate with P₁ within the cavity illustrated in Fig. 4. The Mg²⁺ binding site of NMDMC is made up of Asp¹³³ and P₁, and these negative charges provide the charge balance for the ion. The remaining four co-ordination points of the Mg²⁺ (27) are most likely provided by backbone carbonyl groups, the 3'-hydroxyl of NAD, and water. These interactions place the Mg²⁺ within a box with corners at the carbonyl group of Asp¹³³, the P₁, the carbonyl of Arg₁⁶⁶, and the 3'-hydroxyl of NAD. The position of Mg²⁺ suggests that the ion stabilizes the position of P₁ and NAD in the binding site through hydrogen bonds and charge interactions. The P₁ stabilization role in NMDMC is similar to the role of Mg²⁺ in many proteins that use ATP or other phosphorylated substrates or intermediates (33).

Similarities to P₁ and Mg²⁺ Usage in GR and Rat 3'-Phosphoadenosine 5'-Phosphate/Inositol 1,4-Bisphosphate Phosphatase—The use of P₁ to help bind NAD in NMDMC is similar to NADH binding to native glutathione reductase (GR; Ref. 34). GR preferentially uses NADPH to reduce oxidized glutathione to glutathione. NADPH binds to GR through interactions with two arginines (218 and 224). GR can also use NADH as a cofactor; however, the affinity of GR for NADH is roughly 60-fold weaker than the affinity for NADPH, and it can only bind to the protein in the presence of P₁ (34). The P₁ in GR binds roughly in the same position as the 2'-phosphate of NADPH, interacting with the two arginine side chains. Although the use of the two arginines is common between GR and NMDMC, these motifs are not structurally related; it is not possible to overlay these motifs in the structures. In GR the arginines are independent of the GXGXXG motif (residues 174–179) that interacts with the diphosphate moiety of NAD(P)H (35), whereas in NMDMC Arg₁⁶⁶ is the second residue of the motif. GR has been mutated by Scrutton et al. (36) to preferentially use NADH. In that protein seven residues, including the arginines, were mutated to alter the cofactor binding region to more strongly resemble a classic NADH binding site.

The interaction of Mg²⁺ and P₁, with a ligand containing a sugar moiety similar to the crystal structure of the rat 3'-phosphoadenosine 5'-phosphate/inositol 1,4-bisphosphate phosphatase with bound AMP, P₁, and Mg²⁺ ions (37). In this structure three Mg²⁺ ions bind the P₁, which forms hydrogen bonds with the 2'- and 3'-hydroxyls of the ribose moiety.

The Structure of the Cofactor Binding Site Is Similar to That of NADP Sites—When the cofactor binding site of NMDMC is compared with a classic Rossmann NAD binding site and to the NADP binding site of DCS, the role for the ions becomes more apparent (Fig. 5). The classic NAD site has multiple interactions between the cofactor and the protein to enhance cofactor binding. The GXGXXG consensus sequence interacts with the pyrophosphate moiety and maintains close proximity to the cofactor to maximize interactions. In particular, the second glycine in this region is thought to be important for close contact because any side chain at this position would disrupt cofactor binding (35). NADP-binding proteins also typically have a conserved aspartate residue that forms hydrogen bonds with the hydroxyl groups of the adenine ribose of NAD (35, 38). In contrast, NADP binding sites depend on the interaction of the 2'-phosphate of NADP with an arginine side chain (38), as is the case with DCS (8, 10). The GXGXXG consensus sequence is not as strictly conserved in NADP-binding proteins, and the aspartate residue is no longer conserved. NMDMC and DCS share the consensus sequence of GRGXXG (residues 172–178 of DCS and 165–171 of NMDMC). The substitution of the second glycine by serine in this region disrupts the close interactions usually required for NAD binding (Fig. 5). NMDMC also lacks the conserved aspartate residue. Thus, the cofactor binding site of NMDMC more closely resembles an NADP binding site than a classic NAD binding site. Mutagenesis experiments on DCS showed that NADP binding to the protein is almost entirely dependent on the interaction between the 2'-phosphate and Arg₁⁷³ (8); the other small interactions between the protein and the cofactor were not sufficient for NADP binding. Given the similarity of the NMDMC and DCS cofactor binding sites, it seems clear that the role of the ions in NMDMC is to compensate for the lack of a covalently bound phosphate group on the cofactor. The Mg²⁺ and P₁ ions mediate multiple hydrogen bonding interactions that adapt an NADP site to bind NAD.

Several laboratories have attempted to engineer NADP-specific proteins to preferentially use NAD (36, 39, 40). These groups have used a mutagenesis approach to alter multiple side chains around the cofactor
binding site to mimic the binding site of a homologous protein specific for NAD. Nature has, through evolution, used an entirely different approach to change the cofactor specificity of the mitochondrial methylene-THF dehydrogenase, producing a protein whose specificity for NAD compares favorably to these engineered proteins (36, 39, 40). This use of Mg$^{2+}$ and P to bind NAD to the active site of NMDMC represents a novel variation of the Rossmann fold.

Acknowledgments—We thank Peter Pawelek and Saravanan Sundararajan for helpful discussion.

REFERENCES

1. Christensen, K. E., Patel, H., Kuzmanov, U., Mejia, N. R., and MacKenzie, R. E. (2005) J. Biol. Chem. 280, 7597–7602.
2. Patel, H., Di Pietro, E., and MacKenzie, R. E. (2003) J. Biol. Chem. 278, 19436–19441.
3. Yang, X.-M., and MacKenzie, R. E. (1993) Biochemistry 32, 662–668.
4. Rios-Orlandi, E. M., and MacKenzie, R. E. (1988) Arch. Biochem. Biophys. 278, 19436–19441.
5. Di Pietro, E., Sirois, J., Tremblay, M. L., and MacKenzie, R. E. (2002) J. Biol. Chem. 277, 18703–18709.
6. Di Pietro, E., Sirois, J., Tremblay, M. L., and MacKenzie, R. E. (2002) J. Biol. Chem. 277, 18703–18709.
7. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291.
8. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Mol. Biol. 231, 582–591.
9. NMDMC.

Magnesium and Phosphate Binding Sites of NMDMC

10. Allaire, M., Li, Y., MacKenzie, R. E., and Cygler, M. (1998) Mol. Cell. Biol. 18, 4158–4166.
11. Allaire, M., Li, Y., MacKenzie, R. E., and Cygler, M. (1998) Mol. Cell. Biol. 18, 4158–4166.
12. Allaire, M., Li, Y., MacKenzie, R. E., and Cygler, M. (1998) Mol. Cell. Biol. 18, 4158–4166.
13. Allaire, M., Li, Y., MacKenzie, R. E., and Cygler, M. (1998) Mol. Cell. Biol. 18, 4158–4166.
14. Acharya, S., and MacKenzie, R. E. (1998) Biochemistry 37, 5745–5753.
15. Acharya, S., and MacKenzie, R. E. (1998) Biochemistry 37, 5745–5753.
16. Acharya, S., and MacKenzie, R. E. (1998) Biochemistry 37, 5745–5753.
17. Acharya, S., and MacKenzie, R. E. (1998) Biochemistry 37, 5745–5753.
18. Acharya, S., and MacKenzie, R. E. (1998) Biochemistry 37, 5745–5753.
19. Acharya, S., and MacKenzie, R. E. (1998) Biochemistry 37, 5745–5753.
20. Acharya, S., and MacKenzie, R. E. (1998) Biochemistry 37, 5745–5753.