Several residues lining the ATP-binding site of Methanobacterium thermoautotrophicum nicotinamide mononucleotide adenyltransferase (NMNATase) were mutated in an effort to better characterize their roles in substrate binding and catalysis. Residues selected were Arg-11 and Arg-136, both of which had previously been implicated as substrate binding residues, as well as His-16 and His-19, part of the HXGH active site motif and postulated to be of importance in catalysis. Kinetic studies revealed that both Arg-11 and Arg-136 contributed to the binding of the substrate, ATP. When these amino acids were replaced by lysines, the apparent $K_m$ values of the respective mutants for ATP decreased by factors of 1.3 and 2.9 by factors of 1.9 and 8.8 when the same residues were changed to alanines. All four Arg mutants displayed unaltered $K_m$ values for NMN. The apparent $k_{cat}$ values of the R11K and R136K mutants were the same as those of WT NMNATase but the apparent $k_{cat}$ values of the alanine mutants had decreased. Crystal structures of the Arg mutants revealed NAD$^+$ and SO$_4^{2-}$ molecules trapped at their active sites. The binding interactions of NAD$^+$ were unchanged but the binding of SO$_4^{2-}$ was altered in these mutants compared with wild type. The alanine mutants at positions His-16 and His-19 retained ~6 and 1.3%, respectively, of WT NMNATase activity indicating that His-19 is a key catalytic group. Surprisingly, this H19A mutant displayed a novel and distinct mode of NAD$^+$ binding when co-crystallized in the presence of NAD$^+$ and SO$_4^{2-}$.

Received for publication, May 30, 2003, and in revised form, June 4, 2003
Published, JBC Papers in Press, June 16, 2003, DOI 10.1074/jbc.M205369200

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The atomic coordinates and structure factors (code 1M8F for R11A NMNATase, code 1M8G for R11K NMNATase, code 1M8J for R136A NMNATase, and code 1M8K for H19A NMNATase) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: NMNATase, nicotinamide mononucleotide adenyltransferase; PAPS, adenosine 3′-phosphate 5′-phosphate-sulfate; NAD$, nicotinamide adenine dinucleotide; NaAD$, nicotinic acid adenine dinucleotide; WT, wild type; GCT, glycerol-3-phosphate cytidylyltransferase; NNM$, nicotinamide mononucleotide; NaMN$, nicotinic acid mononucleotide; r.m.s.d., root mean square deviation.
human NMNATases, these bacterial enzymes form dimers or monomers, respectively. Their overall fold, however, is again quite similar.

NMNATase adopts a modified Rossmann-fold with features that place the enzyme in the nucleotidyltransferase superfamily. Other members of this protein family include class I tRNA synthetases, ATP sulfurylase, adenosine 3'-phosphate 5'-phosphosulfate (PAPS) synthase, phosphopantetheine adenyllyltransferase, CTP-phosphocholine cytidylyltransferase, glyceral-3-phosphate cytidylyltransferase (GCT), and pantetheine synthetase (21–27). Besides the nucleotide-binding fold, their common features are a T/HXGH sequence motif at their active sites, and the catalysis of $\alpha,\beta$-phosphodiester bond cleavage of either ATP or CTP leading to mononucleotide cleavage. The importance of the T/HXGH motif can be demonstrated by its absolute conservation in all the proteins that belong to the nucleotidyltransferase superfamily. In a number of members of this enzyme family, including NMNATase, tyrosyl-tRNA synthetase, PAPS synthase, and GCT, the T/HXGH active site sequence motif has been further characterized using structural and mutational techniques (9, 15, 26, 28, 29). The crystal structures of all of these proteins, with the exception of PAPS synthase, have been determined in the presence of their ATP or CTP substrate. In two of three cases the side chain of the first amino acid of the T/HXGH motif was found to interact with the $\beta$-phosphate of ATP (bond distances of 3.1 Å for B. subtilis GCT and 2.8 Å for M. jannaschii NMNATase). The second histidine was bound to the $\alpha$-phosphate of the triphosphate (bond distances of 3.5 Å for B. subtilis GCT and 3.2 Å for M. jannaschii NMNATase) (15, 29). However, the structure of glutaminyl-tRNA synthetases complexed with ATP and tRNA shows that there exists quite a range in the length of these bonds (4.6 Å to the first T/H and 3.5 Å to the second H) (28). Further on, mutagenesis studies (30) revealed that these conserved histidine residues interacted with the ATP moiety solely during the transition state of the reaction, probably providing the increased interaction energy postulated in the transition state complementarity theories of enzyme activity advanced by Haldane (31) and Pauling (32).

An energy-minimized molecule of ATP was modeled into the active site of yeast ATP sulfurylase based on the crystal structure of the protein complexed with product, adenosine 5'-phosphosulfate (23). The authors proposed a novel mode of ATP binding in which the T/HXGH motif interacts with the $\beta$- and $\gamma$-phosphates rather than the $\alpha$- and $\beta$-phosphates of ATP as had been found with the other family members (15, 29).

Given these discrepancies in the way triphosphate binding is envisaged in the various family members, we have applied mutational, structural, and kinetic techniques to further characterize the roles of several active site residues proposed to be important in substrate binding and/or catalysis of NMNATase.

MATERIALS AND METHODS

Site-directed Mutagenesis, Protein Purification, and Crystallization—Site-directed mutagenesis, protein expression, purification, and crystallization were performed as previously described with some minor modifications, mainly involving cell lysis, which now consisted of 5 rounds of sonication (1 min at 50% of maximum output, Branson sonifier 450) (9). Diffraction data were collected from crystals grown in 1.6M (NH$_4$)$_2$SO$_4$, 5% glycerol and 100 mM Tris-HCl, pH 8.0, at 20 °C and flash-frozen with crystallization buffer supplemented with 30% glycerol as cryoprotectant.

RESULTS AND DISCUSSION

Site-directed Mutagenesis

Using the crystal structure of M. thermoautotrophicum NMNATase complexed with NAD$^+$ and a sulfate ion as a guide, target residues were chosen for site-directed mutagenesis aiming to investigate the roles of conserved active site arginine and histidine residues in binding and catalysis of M. thermoautotrophicum NMNATase. The selected residues, Arg-11, His-16, His-19, and Arg-136 are completely conserved in all sequenced archaeal NMNATases.

Probing the ATP-Phosphate Binding Site

**R11K and R136K Mutants**—To be able to evaluate the role of charge as compared with size and shape of a given side chain, the two arginine residues implicated in the binding of the $\gamma$-phosphate of ATP were individually mutated to lysines. The

 steady state kinetic assay—NMNATase was incubated in a solution containing 5 mM MgCl$_2$, 1% ethanol, 1 unit of alcohol dehydrogenase, and 50 mM HEPES buffer, pH 7.5, at 65 °C for 2 min with either varying amounts of NMN$^-$ or ATP while keeping the concentration of the second substrate at 3 mM. The amount of NAD$^+$ formed was measured spectrophotometrically at 340 nm, using alcohol dehydrogenase to convert NAD$^+$ to NADH. The assays with WT, R11K, and R136K NMNATase utilized 2 μg of enzyme, whereas R11A, H16A, H19A, and R136A required 20 μg of enzyme to obtain significant readings. The assays were linear with time and NMNATase concentration. Approximately 5% of the limiting substrate was converted to product.

**Kinetic Data Analysis**—Velocity data obtained by varying one of the substrate concentrations (either ATP or NMN$^-$ using a 1 × 6 matrix) from three independent measurements were averaged and analyzed using Hypero from Cleland’s programs resulting in values for apparent velocity ($V_{\text{app}}$), the apparent Michaelis constant ($K_{\text{m}}$) as well as the apparent first-order rate constant ($k_{\text{cat}}$) and the resulting errors are S.E. (44).

X-ray Data collection and Structure Determination—Diffraction data sets from crystals of NMNATase mutants R11A and R11K were collected at a wavelength of 1.10 Å on beamline X8C (National Synchrotron Light Source, Brookhaven National Laboratory) at 100 K using a Q4 CCD detector (ADSC). Diffraction data sets from a crystal of the R136A mutant were measured at a wavelength of 1.00 Å and at 100 K on beamline BM14C (BioCARS, Advanced Photon Source, Argonne National Laboratory) equipped with a Q4 CCD detector (ADSC). Diffraction data from a crystal of the H19A mutant were recorded on a Rigaku FR-C rotating copper anode equipped with MSC-Yale double mirror optics and a MAR research 345 imaging plate area detector. All x-ray data were processed and scaled with the help of the DENZO/SCALEPACK suite of programs (33). The H19A mutant structure was determined employing the molecular replacement program package AMoRe (34, 35). All other crystals were sufficiently isomorphous with the WT NMNATase to allow the immediate use of the RIGID routine of CNS, version 0.9 (36), to correctly place the search model. Model visualization and rebuilding were done with the program O (37) and CNS, version 0.9, was used for refinement. Water molecules were initially picked using CNS and then manually verified in O using the following criteria: a peak of at least 2.5 σ in an $F_o$ – $F_c$ map, a peak of at least 1.0 σ in a $2F_o$ – $F_c$ map, and reasonable intermolecular interactions. Crystallographic and refinement statistics are found in Tables I and II. The programs MOLSCRIPT (38), RASTER 3D (39), and SPOCK (40) were used in the production of the figures.
Arg-11 and Arg-136 form two interactions each to the
M. jannaschii NMNATase reveal that both
Km values for ATP for both arginine to lysine mutants,
and Arg-136 without causing major changes in the kinetic properties
of NMNATase. We take this as an indication that at these positions the positive charge of the side chain is most important.

The Km values for NMN+ of both mutants were similar to that of WT NMNATase, 0.13 and 0.14 mM for R11K and R136K, respectively, compared with 0.08 mM for WT. Arg-136 does not interact with NMN+; Arg-11, however, forms a long hydrogen bond (3.5 Å) to one of the oxygen atoms of the NMN+ phoshate. It is likely that this interaction contributes to catalysis by aiding in the positioning of the NMN+ phosphate but does not contribute strongly to the binding of NMN+ as reflected in the Km value. Together with a 4-fold decrease of kcat for R11K, this could indicate a minor role for Arg-11 in preparing the NMN+ phosphate for its in-line attack on the α-phosphate of ATP. The shorter length of the lysine side chain and the removal of the bi-dentate potential of an arginine head group could lead to a loss of this support function and might be reflected in the lower kcat of the mutant.
Overall, the kinetic results are consistent with the findings of the crystal structure of NMNATase, in which Arg-11 and Arg-136 are directly involved in binding the γ-phosphate of ATP, the part of this phosphate chain of the substrate that is furthest from the point where the actual chemical reaction happens. Therefore, one would expect to see the most pronounced change in parameters describing ATP binding and only minor effects on catalytic roles.

**R11A and R136A Mutants**—Given the very modest changes seen upon replacing Arg-11 and Arg-136 with lysine, more drastic modifications were introduced by constructing the corresponding alanine mutants. ATP binding to NMNATase was now affected more strongly with changes in both the $k_{cat}$ and the $K_m$ values for ATP. For R11A, we measured a 20-fold reduced $k_{cat}$ value of 0.5 s$^{-1}$ compared with 10 s$^{-1}$ for WT. The increase in the $K_m$ value for ATP was 2-fold to 0.30 mm versus 0.16 mm for WT. The $k_{cat}/K_m$ value decreased 37-fold to 1.7 ms$^{-1}$ s$^{-1}$ compared with 63 ms$^{-1}$ s$^{-1}$ for WT. In the case of the R136A mutant, a $k_{cat}$ value of 0.4 s$^{-1}$ was measured, a 25-fold decrease when compared with 10 s$^{-1}$ for WT. The $K_m$ value increased 9-fold to 1.4 mm from 0.16 mm for WT resulting in a $k_{cat}/K_m$ value of 0.3 ms$^{-1}$ s$^{-1}$, a 210-fold decrease compared with 63 mm$^{-1}$ s$^{-1}$ for WT. The much higher Michaelis-Menten constants seen in both mutants reflect the relative contributions of these arginine residues to the binding of ATP by NMNATase. Although the catalytic rates were somewhat reduced for both mutants, it is clear that these positively charged amino acids support triphosphate binding but are not absolutely essential for NMNATase catalysis.

**H16A and H19A Mutants of the “Fingerprint” Motif**—The roles of the two His residues within the THXGH active site sequence motif have been well characterized for several members of the nucleotide transferase superfamily. In GCT the motif is $^{14}$HWGH$^{17}$ and replacement of either of the two histidine residues by alanine almost completely abolished catalytic activity; $V_{max}$ values were decreased by factors of $5 \times 10^5$ and $4 \times 10^4$ for His-14 and His-17, respectively, without affecting the $K_m$ values, indicating a sole role in catalysis and none in CTP binding (27). Introducing the same changes to the histidine residues of the $^{42}$HNGH$^{428}$ motif in human and in murine PAPS synthase produced enzymes that did not show any catalytic activity in cellular extracts (24). Identical mutations in CTP-phosphocholine cytidylyltransferase again resulted in inactive and unstable enzyme (26). In contrast to these reports, our results for the H16A mutant showed that *M. thermoautotrophicum* NMNATase is still able to catalyze the synthesis of NAD$^+$. The catalytic rate was only reduced by a factor of 17 (Table III) ruling out a critical role for this residue in *M. thermoautotrophicum* NMNATase catalysis. Together with a 7.5-fold increase in $K_m$ for ATP, this modification diminishes the catalytic efficiency of the enzyme 126-fold. The H19A mutant also possessed NMNATase activity but at a 77-fold reduced rate. Quite surprisingly, when the $K_m$ value for ATP was measured it deviated from the Michaelis-Menten parameters determined for the other *M. thermoautotrophicum* NMNATase enzymes. From Table III, it can be seen that the $K_m$ for ATP has decreased 20-fold to 0.008 mm compared with 0.16 mm for WT. The $K_m$ for NMN remained the same. The $k_{cat}/K_m$ value for ATP does not reflect the expected decrease because the observed $K_m$ value (0.008 mm) indicates increased affinity for ATP by H19A NMNATase. We have previously shown that the structure of H19A NMNATase contained a trapped molecule of NMM$^+$ indicating inability to bind ATP and catalyze biosynthesis of NAD$^+$ at 24 °C (the growth temperature of the *E. coli* culture after induction). Therefore, the kinetic results obtained for the H19A mutant lead us to speculate that the residual catalytic activity may be caused by a minor contamination in our enzyme preparation. Potential culprits could be the products of the *E. coli* NadD or NadR genes or another *M. thermoautotrophicum* NMNATase mutant at the His-19 position resulting from an error during protein translation (41).

**Structural Analysis**

**Crystallization and Structure Determination of R11A, R11K, and R136A**—Crystals of NMNATase arginine mutants grew under conditions similar to those established for WT NMNATase and with almost identical crystal morphologies. Consistently, however, their dimensions were smaller and they were more fragile. This was accompanied by diffraction patterns of lower resolution and reflections with significantly higher mosaicity. The crystal structures of R11A, R11K, and R136A NMNATase were determined at resolutions ranging from 2.4 to 2.0 Å. Only crystals too small for meaningful data collection were obtained for the R136K mutant.

The atomic models constructed are of good quality as indicated by low R-factors and small deviations from ideal bond length and bond angle parameters (Table II). According to PROCHECK, between 91.6 and 93.0% of the residues are in the most favored regions of the Ramachandran plot and the remaining residues occupy additionally allowed regions (42). No residues are found in the generously allowed or disallowed regions.

All arginine mutant enzymes crystallized with one subunit of the hexameric structure in the asymmetric unit, the same arrangement as in crystals of WT NMNATase (9). Continuous, interpretable electron density was observed for residues 4 to 170. The overall structures of the R11A, R11K, and R136A mutants are quite similar to that of WT. 167 equivalent Ca atoms of each mutant monomer superimpose to their equivalents in WT with r.m.s.d. values of 0.26 Å for R11A, 0.29 Å for R136A, and 0.12 Å for R11K. The relative arrangement of monomers in the hexamer is also unchanged for all mutant structures. None of the mutations resulted in any gross structural changes in the overall structure of the protein.

The crystal structures of R11A, R11K, and R136A NMNATase all show a molecule of NAD$^+$ in the active site, as had been found for WT (Fig. 1) (9). Because all of these mutants had the capacity to catalyze the coupling of NMM$^+$ and ATP to
NAD\textsuperscript{+}, it was not very surprising to find electron density corresponding to a product molecule in the active site. The NAD\textsuperscript{+} molecules bound to the three mutants superimpose quite well with each other and with the one found in WT NMNATase (Fig. 1). Except for the obvious changes introduced by mutagenesis, all dinucleotide-protein interactions described for WT NMNATase are also seen in the mutant enzymes. The nicotinamide moiety of NAD\textsuperscript{+} interacts with the side chains of Ser-39, Asp-80, Gln-84, and Asn-105 and stacks with Trp-87, whereas the adenyl moiety of NAD\textsuperscript{+} interacts with His-19 and the backbone of Gly-104, Phe-125, and Tyr-130 (Fig. 1).

There is, however, one distinct change between WT and mutants. Previously we had identified a sulfate ion occupying the same site as the γ-phosphate position of ATP (9). The crystal structure of R11A does not show such a sulfate ion at the substrate-binding site. The binding of the ATP molecule as a whole is only slightly affected by the mutation, as seen by the $K_m$ measured for ATP. Binding of a single sulfate ion at the γ-phosphate position, however, was abolished by the loss of its interactions with the positively charged side chain.

Although replacing an arginine with a lysine residue seems a most conservative change, conserving the positive charge and a side chain of almost comparable length, the crystal structure of the R11K mutant shows that the affects of this mutation can be more dramatic in the case of $M$. thermoautotrophicum NMNATase. The side chain of R11K has moved away from the γ-phosphate position, however, is drastically different (Figs. 2 and 3).

Removing the charged side chain of Arg-11 (by mutation to Ala or reorientation of the lysine chain in R11K) abolishes both the Β- and γ-phosphate binding sites as it removes a crucial, energetically favorable interaction for both sites. Consequently, no sulfate ion binding is observed in Arg-11 mutants. The loss of Arg-136, however, is of significance only for the γ-phosphate site; a sulfate ion can still be bound at the β-phosphate site. Whereas in WT NMNATase the γ-phosphate site seems to display the higher affinity for phosphate groups or their mimics, sulfate ions, this role seems to be reversed in the R136A mutant.

Crystallographic contacts formed between adjacent WT hexamers compared with the sulfate ion in WT NMNATase. It now mimics the β-phosphate of ATP instead of the γ-phosphate (Fig. 1). For this mutant, the $K_m$ value for ATP was increased 15-fold, again indicating a lowered affinity for ATP. In the $M$. jannaschii NMNATase-ATP complex, the corresponding arginine residue interacts with the γ-phosphate of the nucleotide only (15).

Crystallization and Structure Determination of H19A Co-crystallized with NAD\textsuperscript{+}—As purified from $E$. coli, H19A had crystallized with a molecule of NMN\textsuperscript{+} and a sulfate ion at the γ-phosphate position (9). To provide a common structural background against which the effects of various mutations in the ATP-binding site could be compared, NMN\textsuperscript{+} was exchanged for NAD\textsuperscript{+}. Crystals of the NAD\textsuperscript{+} complex of the H19A mutant grew under conditions similar to those established for WT NMNATase crystals. However, they displayed a different crystal morphology, chunky, distorted bipyramids rather than the usual hexagonal rods, accompanied by a change in space group from the standard P6\textsubscript{3}2\textsubscript{2} to P3\textsubscript{1}21. The structure of H19A complexed with NAD\textsuperscript{+} was determined to 3-Å resolution. The asymmetric unit contained a trimer of NMNATase molecules, one-half of the hexamer found in solution. The internal packing of the six subunits into the hexamer is identical in the two space groups, the packing of hexamers against each other, however, is drastically different (Figs. 2 and 3).

The atomic models corresponding to these three molecules are of good quality. According to PROCHECK, 92.4% of the residues are in the most favored regions of the Ramachandran plot and the remaining residues occupy additionally allowed regions with no residues in the generously allowed or disallowed regions (42). The crystal packing of the NAD\textsuperscript{+} complex of H19A (Fig. 3) is different from that of the same complex of WT NMNATase (Fig. 2). The crystal contacts in the former complex

![Crystal Structures of NMNATase Mutants](image-url)
are formed between residues 127 and 130 of one subunit (either molecule A or C) with residues 28–30 of an adjacent subunit (molecule C or A, respectively). Backbone interactions are also formed between residues 123 and 127 of one subunit (molecule A or C) with the same residues on an adjacent subunit in an antiparallel fashion (Fig. 3). In all cases the interactions are formed between molecules A and C. Molecule B only forms intra-hexamer contacts; it does not form any inter-hexamer contacts in the crystal lattice. In contrast, the crystal contacts for WT NMNATase complexed with NAD$^+$ are formed between residues 146 and 167 from an adjacent hexamer (Fig. 2). Unfortunately, we were unable to obtain any crystals for the H16A mutant.

The overall structure of the H19A mutant was quite similar to that of WT. 167 equivalent Cα atoms of H19A superimpose to their equivalents in WT with r.m.s.d. values of 1.1–1.2 Å. To our surprise, the crystal structure of the H19A-NAD$^+$ complex revealed a new mode of nucleotide binding. The position and orientation of the adenylly moiety of NAD$^+$ in the active site of H19A NMNATase is quite different from that found in all other structures of NMNATases determined to date (Fig. 4). In this new conformation, the nicotinamide ring is the only part of NAD$^+$ that retains its position. The neighboring ribose rotates slightly and shifts toward helix 4 (9). The NMN$^-$-phosphate moves closer to helix 4 but still interacts with Asn-105. The AMP-phosphate approaches the backbone of Arg-11 and forms a bond with its amide. The AMP-ribose has changed orientation by $-140^\circ$ in the plane of the ring. The N-6 atom of the adenine ring now interacts with the backbone carbonyl of Leu-124 and the aromatic base forms a favorable stacking interaction with the side chain of Tyr-126. In the structure of WT NMNATase, this side chain is very mobile, pointing in the opposite direction into solvent. This surprise finding reveals a remarkable adaptability of an active site whose main catalytic effect is thought to be the proper placing of the reaction partners.

In a first effort to better understand the chemistry of the NMNATase reaction as well as that of nucleotidytransferases in general, site-directed mutants of residues clustered around the binding sites of the three ATP-phosphates were generated and probed kinetically as well as structurally. Surprisingly, all of the (Arg to Ala, Arg to Lys, and His to Ala) mutants were still catalytically active. This is in stark contrast to the results obtained with the enzymes PAPS synthase, CCT, and GCT where Ala mutants of the H$X_H$ motif completely abolished nucleotidytransferase activity (27, 43). Clearly, the T/H$X_H$ sequence motif, found in all members of this nucleotidytransferase superfamily, is important in NTP binding and transition state stabilization or else it would not have been as well conserved from Archaea to humans. Based on crystal structures of various tRNA synthetases as well as mutagenesis studies of the T/H$X_H$ motif, Veitch and colleagues (26) postulated that the sequence T/H$X_H$ is a general motif for ATP binding and stabilization but that the exact role of the histidine residues in each enzyme can vary. An example supporting this idea is yeast ATP sulfurylase, where modeling an energy-minimized molecule of ATP into the active site of this enzyme led Ullrich et al. (23) to propose that the T/H$X_H$ motif bound to the $\beta$- and $\gamma$-phosphates instead of the $\alpha$- and $\beta$-phosphates as in most other members of this protein family. The results described here argue that the histidine residues in the T/H$X_H$ motif are not essential for the stabilization of the transition state in the NMNATase reaction. First, this may seem surprising but the
mechanism proposed for NMNATase might help to explain this finding. It is thought that the enzyme accelerates the synthesis of NAD$^+$ almost purely by providing a binding template, orienting the reacting partners ATP and NMN$^-$ in ideal positions for nucleophilic attack to occur. No involvement of acid/base chemistry or nucleophiles, other than the phosphate groups of the substrate themselves, have been implicated in catalysis. Therefore, a moderate loss in binding energy does not automatically translate to a reduction of catalytic rate because there remain a sufficient number of residues lining the two substrate-binding sites to ensure proper orientation of the reaction partners. The kinetic constants obtained in this study confirm that the two residues, Arg-11 and Arg-136, are involved in binding the γ-phosphate of ATP but do not have a major effect on $k_{\text{cat}}$. His-16 is involved in binding the β-phosphate of ATP. His-19 plays a distinct role in catalysis because its change to an alanine resulted in a significant reduction in $k_{\text{cat}}$. It is, however, not absolutely crucial as in the other members of the family.

In the crystal structure of B. stearothermophilus tyrosyl-tRNA synthetase complexed with ATP, a complex that reflects the ground state of the reaction, there are no interactions between His-45 and the bound ATP molecule. His-48 forms a hydrogen bond to the 5′-oxygen of ribose. Based on difference energy diagrams obtained from mutagenesis studies, Fersht (30) concluded that the histidine residues in the 45HIGH48 motif interact with the γ-phosphate and the ribose 5′-oxygen during the transition state of the reaction. Clearly this picture
is quite different from the one observed for the ATP complex of *M. jannaschii* NMNATase (29). Here, the two histidine residues of the NMNATase $^{13}$HWH$^{16}$ motif directly interact with the $\alpha$- and $\beta$-phosphates of ATP in the ground state, His-13 with the $\beta$-phosphate and His-16 with the $\alpha$-phosphate. Also in contrast to what was observed by Fersht (30) in the E-Tyr-AMP product complex in which His-48 is still bound to the ribose 5'-oxygen, in the NAD complex of *M. thermoaotrophiucm* NMNATase (9), His-19 is interacting with an oxygen atom of the AMP-phosphate, which corresponds to the $\alpha$-phosphate of the substrate ATP.

Interestingly, co-crystallization and structure determination of H19A with NAD$^+$ demonstrated that the missing interaction between His-19 and one of the oxygen atoms of the AMP-phosphate was sufficient to hinder binding of NAD$^+$ in the same orientation as observed in WT NMNATase. Obviously, the enzyme has the capacity to reorganize its structure such that it is able to bind NAD$^+$ in a completely different conformation. Therefore, it is not difficult to imagine that the enzyme can tolerate substitution of either of the His residues within the HXGH motif by re-organizing its active site in a way that still enables the catalysis of NAD$^+$ synthesis.

Acknowledgments—We thank all staff members of BioCARS for help during data collection at Sector 14 of the Advanced Photon Source as well as Leon Flaks and Joel Berendzen for help during data collection at beamline X8C of the National Synchrotron Light Source. We are also grateful to Joanne Turnbull (Concordia University) and Thomas Ley (Albert Einstein College of Medicine, Yeshiva University) for helpful discussions.

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