Structure of Human Nicotinamide/Nicotinic Acid Mononucleotide Adenylyltransferase

BASIS FOR THE DUAL SUBSTRATE SPECIFICITY AND ACTIVATION OF THE ONCOLYTIC AGENT TIAZOFURIN*

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Nicotinamide/nicotinate mononucleotide (NMN/NaMN)/adenyltransferase (NMNAT) is an indispensable enzyme in the biosynthesis of NAD† and NADP‡. Human NMNAT displays unique dual substrate specificity toward both NMN and NaMN, thus flexible in participating in both de novo and salvage pathways of NAD synthesis. Human NMNAT also catalyzes the rate-limiting step of the metabolic conversion of the anticancer agent tiazofurin to its active form tiazofurin adenine dinucleotide (TAD). The tiazofurin resistance is mainly associated with the low NMNAT activity in the cell. We have solved the crystal structures of human NMNAT in complex with NAD, deamido-NAD, and a non-hydrolyzable TAD analogue β-CH2TAD. These complex structures delineate the broad substrate specificity of the enzyme toward both NMN and NaMN and reveal the structural mechanism for adenylation of tiazofurin nucleotide. The crystal structure of human NMNAT also shows that it forms a barrel-like hexamer with the predicted nuclear localization signal sequence located on the outside surface of the barrel, supporting its functional role of interacting with the nuclear transporting proteins. The results from the analytical ultracentrifugation studies are consistent with the formation of a hexamer in solution under certain conditions.

Nicotinamide/nicotinic acid mononucleotide (NMN/NaMN)† adenylyltransferase (NMNAT) is an indispensable enzyme in both de novo and salvage pathways of NAD biosynthesis (1, 2) (Fig. 1a). In human, the de novo NAD biosynthesis proceeds via several enzymatic steps to transform tryptophan into NaMN, which is then converted into deamido-NAD (NaAD) via the amidation step catalyzed by NAD synthetase (Fig. 1a). Human NMNAT displays unique dual substrate specificity toward both NMN and NaMN (3, 4) and is thus capable of participating in both de novo and salvage pathways of NAD generation.

In addition to the fundamental role as coenzyme in hundreds of oxidation-reduction reactions throughout the cell, NAD can also be used as a substrate for the modification of a variety of nuclear proteins by ADP-ribosyltransferases (5, 6) and for the repair of DNA by DNA ligase in bacteria (7, 8). The formation of protein-coupled poly(ADP-ribose) relaxes the chromatin structure and facilitates DNA regulatory and repair events under conditions of DNA damage. Recently, several NAD derivatives such as cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate have been reported to be potent intracellular calcium-mobilizing agents participating in calcium-dependent signaling pathways (5, 9). In some situations these processes may significantly deplete the cellular NAD pool which, coupled with a decrease in ATP production, could potentially lead to a cellular energy crisis culminating in cell death. It is thus crucial that NAD biosynthesis is actively regulated, and proper NAD levels are maintained. Human NMNAT (hsNMNAT) has been shown to locate exclusively within the nucleus (10, 11), exhibit specific inhibitory interactions with poly(ADP-ribose) polymerase (11, 12), and can be phosphorylated by nuclear protein kinase(s) (11). These features suggest that in addition to its critical biosynthetic function, human NMNAT can be a subject of complex regulation and thus may play a regulatory role in cellular NAD metabolism. Moreover, human NMNAT catalyzes the rate-limiting step of the metabolic conversion of the anticancer agent tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) to its active form tiazofurin adenine dinucleotide (TAD) (13) (Fig. 1b). TAD is a potent inhibitor of inosine monophosphate dehydrogenase, a rate-limiting enzyme in the de novo biosynthesis of guanidine nucleotides, including GTP and dGTP. Inhibition of inosine monophosphate dehydrogenase produces an overall reduction in guanine nucleotide pools, which leads to the interruption of DNA and RNA synthesis, and compromises the ability of G proteins to function as transducers of intracellular signals (14). Resistance to tiazofurin relates mainly to a decrease in NMN adenylyltransferase activity (15).

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† The abbreviations used are: NMN, nicotinamide mononucleotide; NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide; TAD, tiazofurin adenine dinucleotide; NLS, nuclear localization signal; hsNMNAT, human NMNAT.
Human MNM/NMT belongs to the same nucleotidyl transferase superfamily as the bacteria and archaea enzymes but shares only limited sequence identity (<20%). They also differ in quaternary structure and biochemical and enzymatic properties. To gain an insight into the mechanism of human MNM/NMT substrate recognition, we solved the crystal structures of human MNM/NMT bound to NAD, NaAD, and a non-cleavable TAD analogue, β-methylene-TAD (β-CH₂-TAD) (16), respectively. A comparative analysis of these complexes reveals the structural basis for the unique dual specificity of the enzyme toward both NMN and NaMN. Extra interactions are found between β-CH₂-TAD thiazole atoms and the enzyme, which might result in increased affinity of the ligand. Our structural data show that human MNM/NMT forms a barrel-like hexamer, with the predicted nuclear localization signal (NLS) sequences located on a flexible loop at the surface of the barrel where it is potentially able to interact with the nuclear transporting proteins.

**EXPERIMENTAL PROCEDURES**

Protein Overexpression and Purification—The gene encoding hSNMNAT (GenBank™ accession number AR092665) was amplified from a human brain cDNA library (CLONTECH) by PCR and was cloned into a PET-derived vector containing a T7 promoter, His₆ tag, and a TEV protease cleavage site (gift from Dr. Meg Phillips, University of Texas Southwestern Medical Center). The resulting plasmid was transformed into the Escherichia coli strain BL21(DE3) (Invitrogen) for expression. The overexpressed human MNM/NMT protein was first purified with a nickel-nitrilotriacetic-agarose (Qiagen) column, followed by a Mono S (Amersham Biosciences) and a Superdex 200 gel filtration column (Amersham Biosciences). The selenomethionine hSNMNAT was expressed in the met’ auxotrophic strain B834(DE3) (Novagen) grown in minimum media, supplemented with selenomethionine and other nutrients (17), and was purified using the same procedure as the native protein.

Crystallographic and Data Collection—The hSNMNAT crystals were grown at 20 °C using the hanging drop vapor diffusion method. 25 mg/ml hSNMNAT in 100 mM Hepes, pH 7.2, 0.5 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA, and 0.03% Brij-35 (Buffer A) was first incubated with 10 mM NAD and then mixed with an equal volume of the reservoir solution Buffer B (0.1 mM sodium acetate, pH 4.2–4.6, 1.6–1.8 mM sodium formate) and equilibrated against the reservoir. Large crystals appeared after 1–2 weeks and were grown to a maximal size of 0.9 x 0.5 x 0.2 mm³ in a month. These hSNMNAT-NAD co-crystals belong to the P2₁ space group with cell dimensions a = 130.8 Å, b = 90.7 Å, c = 136.7 Å, β = 117.0°, and diffract to 2.4 Å resolution on a rotating anode x-ray generator. The co-crystals of hSNMNAT with NaAD or β-CH₂-TAD were grown under the same conditions with the addition of 5 mM of the appropriate ligand in the protein solution. These crystals are isomorphous to the hSNMNAT-NAD complex crystals.

For data collection at 100 K, crystals were transferred stepwise to a cryoprotection solution containing Buffer B and additional 30% glycerol. A 2.2-Å resolution data set was collected from a xenon-treated hSNMNAT-NAD complex crystal at beamline X12B, National Synchrotron Light Source, Brookhaven National Laboratory. Although this xenon-treated crystal did not yield useful phase information, we used this data set as the native data set in the final refinement because of its superior quality over other crystals. A two-wavelength selenomethionine multiwavelength anomalous diffraction data set was collected at beamline 19ID of Advanced Photon Source, Argonne National Laboratory. The data sets from hSNMNAT-NAD and hSNMNAT-TAD crystals were collected on a RAXIS IV image plate detector equipped

**TABLE I**

| selenomethionine MNM/NMT | NAD complex | NaAD complex | β-CH₂-TAD complex |
|---------------------------|-------------|--------------|-------------------|
| Resolution (Å)           | 2.3         | 2.3          | 2.2               |
| Total observation         | 1,010,642   | 507,730      | 435,916           |
| Unique reflection         | 127,331     | 127,801      | 132,989           |
| Completeness (outer shell)| 100% (99.8%)| 99.9% (99.7%)| 91.8% (84.8%)     |
| Rsym (outer shell)⁺       | 0.090 (0.416)| 0.070 (0.400)| 0.080 (0.465)    |
| Lp (outer shell)          | 24.5 (5.0)  | 19.4 (3.2)   | 12.9 (1.9)       |
| Figure of merit           | 0.40        | 0.40         | 0.40              |
| Refinement                |             |              |                   |
| Rwork                     | 21.8%       | 21.0%        | 20.4%             |
| Rfree                     | 25.0%       | 24.6%        | 23.2%             |
| Protein atoms             | 11,166      | 11,166       | 11,166            |
| Hetero groups             | 6NAD, 12Xe  | 6NaAD        | 6TAD              |
| Water molecules           | 747         | 481          | 627               |
| R.m.s.d. bond length      | 0.014Å      | 0.012Å       | 0.014Å            |
| R.m.s.d. bond angle       | 1.60°       | 1.53°        | 1.70°             |

⁻ R_free = Σ||Fo|| - |Fc||/Σ|Fc||, where Fo and Fc are the observed and calculated structure factors, respectively.
³ Rwork is the R factor calculated for a randomly selected 5% of the reflections that were omitted from the refinement.
⁴ R.m.s.d., root mean square deviation.
with a Rigaku RU-H3R generator (MSC, The Woodlands, TX) and Osmic mirrors. All diffraction data were processed and scaled with the HKL2000 package (18). The statistics for all data sets are listed in Table I.

Phasing and Refinement of hsNMNAT Complexes—The initial phases of the hsNMNAT crystal structure were solved by the selenium multiwavelength anomalous diffraction phasing method using the K-edge anomalous signal of the selenium atoms. There are total of 4 methionines in the hsNMNAT monomer (279 residues) including the N terminus Met. The heavy atom search routine in CNS (19) found all 18 internal selenium sites on the data collected at the peak wavelength. The heavy atom parameters were then refined, and the phases were calculated in CNS. The overall figure of merit is 0.40 between 20 and 2.3-Å resolution. Density modification, including solvent flipping in CNS, resulted in a much improved map, and a model was built into the resulting density using O (20). We located six hsNMNAT monomers in the asymmetric unit and they form a hexamer.

The refinement of the hsNMNAT-NAD complex structure was carried out in CNS (19) with 6-fold non-crystallographic symmetry restraints. The first round of refinement resulted in an $R_{\text{work}}$ of 31.2% and $R_{\text{free}}$ of 31.8%, and the density for the NAD molecule was evident in the resulting electron density map. A model for the bound NAD was derived from the coordinates for NaAD in the *E. coli* NMNAT-NaAD complex structure (21) and was included in the subsequent rounds of refinement. The non-crystallographic symmetry restraint was relaxed in the final rounds of refinement which resulted in the improvement of both $R_{\text{work}}$ and $R_{\text{free}}$. The refinements of hsNMNAT complexed with $\beta$-CH$_2$-TAD and NaAD were carried out using a similar procedure. The $\beta$-CH$_2$-TAD coordinates and its topology and structure parameter files were
Analytical Ultracentrifugation Studies—Sedimentation equilibrium experiments were performed with a Beckman XL-A analytical ultracentrifuge and using both scanning absorption and Rayleigh interference optics at a wavelength of 280 nm. We used an An-60Ti rotor with double sector cells of 1.2-cm path length at 4°C for all experiments. The sedimentation equilibrium was carried out at 9,000 and 11,000 rpm. The Rayleigh interference optics was used for samples of 5 mg/ml in Buffer A (100 mM Hepes, pH 7.2, 0.5 M NaCl, 2 mM dithiothreitol, 1 mM EDTA, and 0.03% Brij-35) in the absence and presence of NAD. The scanning absorption optics was used for the sample of 0.3 mg/ml in the same buffer in the absence of NAD. All centrifugation data were analyzed with the Optima™ data analysis software.

RESULTS AND DISCUSSION

Overall Architecture of hsNMNAT Monomer—In the final refined hsNMNAT complex structures (Fig. 2a), the first five residues together with the N-terminal His<sub>6</sub> tag fusions (for a total of 20 amino acids), as well as four residues at the C terminus (276–279), are disordered. Additionally, a stretch of 37 residues between helix C and strand d (from 109 to 146) is also disordered in the crystal. This region contains the predicted nuclear localization signal (NLS) sequence PGRKRKW and two putative phosphorylation site serine residues (Ser-109 and Ser-136) (11). Sequences corresponding to this region do not exist in bacterial or archaeal NMNATs (Fig. 2b).

The hsNMNAT monomer structure contains a central six-stranded parallel β-sheet flanked by several helices (Fig. 2a). This is different from the E. coli and archaeal NMNATs (21, 23, 24), which contain a seven-stranded and a five-stranded central β-sheet, respectively (Fig. 2a). Additionally, hsNMNAT has a C-terminal extension that contains residues 255–279. The last short helix (residues 267–274) is located in the close proximity of the pyridine-binding site and may play a role in the substrate recognition (see below).

The Organization of hsNMNAT Hexamer—Human NMNAT in crystal forms hexamer with a 32-point group symmetry and can be viewed as a trimer of dimers (Fig. 3, a and b). The dimer interface buries a surface area of 1100 Å² per each monomer. Half of these contacts are from hydrophilic interactions between polar atoms. In particular, the main chain amide and carbonyl groups of residues 218–220 are hydrogen-bonded with the same main chain groups from the other monomer in the dimer, resulting in a short stretch of a two-stranded antiparallel β-sheet. The interactions between dimers in the hexamer appear less extensive. Upon formation of the hexamer, a total of about a 2000-Å² surface area of the dimer is buried due to the cooperative interactions with the two adjacent dimers. Approximately 60% of these contacts are between polar atoms.

Because hsNMNAT forms a hexamer in the crystal as opposed to the previously reported tetramer as determined by gel filtration studies (3) (Fig. 3), we performed analytical ultracentrifugation studies in order to characterize the oligomerization state of the protein in solution. The results indicate that at 5 mg/ml concentration, the enzyme exclusively forms a hexamer with an apparent mass of 197 kDa. Binding of NAD does not change these results. However, at lower protein concentration (0.3 mg/ml), the apparent molecular mass was 152 kDa, which is between the calculated mass of a hexamer (192 kDa) and a tetramer (128 kDa). The most straightforward interpretation of
the data would be that there exists a hexamer-dimer equilibrium in solution. However, our data cannot rule out a possible hexamer-dimer-tetramer equilibrium.

The hsNMNAT hexamer has an appearance of a closed barrel with rugged rims (Fig. 3). The outer diameter of this barrel is about 90 Å and the inner channel about 25 Å across. The active sites of the enzyme are accessible from inside this central channel. The disordered region between helix C and strand d containing the predicted NLS sequences is located at the outer surface of the barrel and would be able to interact with the nuclear transporting proteins to cross the nuclear pore. The location and apparent conformational flexibility of this region is characteristic of NLS sequences. An electrostatic potential calculation of the hexamer reveals that there is a cluster of positively charged residues at the dimer-dimer interface, resulting in a strongly positively charged patch that appeared only upon hexamerization (Fig. 3c). Because these positively charged residues (Arg-40, Lys-205, and Arg-207 from monomer A, Arg-228, Arg-231, and Arg-232 from monomer B, and the same residues from the second dimer C–D) come from all four adjacent monomers, this surface electrostatic feature does not exist with the monomer or dimer. We speculate that factors that help to neutralize this patch of positive charges would facilitate hexamer formation. The fact that all human NMNAT crystals obtained so far have been grown in acidic buffer at pH 4.2–4.6 supports this notion. Inside the cell nucleus, phosphorylation at the outer disordered loop or interaction with negatively charged poly(ADP-ribose) might also facilitate the hexamerization. One of the predicted phosphorylation sites (Ser-256) is close to this positively charged patch. On the other hand, formation of the NMNAT hexamer may generate specific interaction surfaces that could interact with other proteins, such as poly(ADP-ribose) polymerase or protein kinases. It may also increase the affinity of hsNMNAT to the highly negatively charged poly(ADP-ribose) or nucleic acid. At present the nuclear protein kinase that may phosphorylate human NMNAT has not been identified, and the exact phosphorylation sites on human NMNAT have yet to be determined. Further studies are required to learn how phosphorylation might affect the structure and function of human NMNAT and how human NMNAT interacts with other proteins including poly(ADP-ribose) polymerase and specific protein kinases.

Interactions with NAD and NaAD—hsNMNAT displays dual substrate specificity toward both NMN and deamidated NaMN. To learn how hsNMNAT recognizes both carboxamide and carboxylate groups on the pyridine ring of the mononucleotides, we have solved the structures of both complexes of hsNMNAT with NAD and NaAD. In the hsNMNAT-NAD complex structure, the conformation of the enzyme in the active site is virtually identical to that of Fig. 4. Active site of human NMNAT. a, stereo diagram of the active site of human NMNAT with bound NAD. The bound NAD and relevant protein residues are shown in ball-and-stick representation. The hydrogen bonds are indicated by dotted lines. The one-directional arrow indicates the change in position of water H2 in the two complexes. The two-ended arrow indicates the disruption of the contact between the Leu-168 carbonyl and the NaAD carboxylate group.
in the NAD complex. The conformation of the bound NaAD is also very similar to that of bound NAD, except that the pyridine ring is moved slightly toward helix C and away from the carbonyl oxygen of Leu-168 by $-0.6$ Å (Fig. 4b). The Leu-168 carbonyl also appears to move slightly away from the bound pyridine by $-0.3$ Å. This resulted in the distance between the second oxygen of NaAD carboxylate and Leu-168 main chain carbonyl oxygen to become 4.0 Å, well beyond the range of contact. Additionally, a highly conserved water molecule $\omega 4$ in the enzyme-NAD complex “moved” about 2 Å toward Leu-168 in the NaAD complex and is now hydrogen-bonded to the carbonyl of Leu-168 (3.0 Å). This interaction would compensate for the partial negative charge of the Leu-168 carbonyl oxygen and thus favor the binding of the carboxylate group of NaMN. In the enzyme-NAD complex, the water molecule $\omega 4$ is held in place by the side chains of Glu-94 (2.8 Å) and Asn-273 (2.7 Å) and is invariant in all six protomers in the hexamer. Upon binding the deamido-NAD and “moving” closer to the Leu-168 carbonyl and NaAD carboxylate (3.2 Å), $\omega 4$ lost its contact with Glu-94 but is still coordinated by Asn-273. This change in position of $\omega 4$ is observed in all six human NMNAT-NaAD monomers in the asymmetric unit, indicating that this effect is not a crystallization artifact. Notably, Asn-273 is located on the last short helix near the C terminus of enzyme. This C-terminal structural motif is unique in the hsNMNAT and is not present in the bacterial and archaeal enzymes. The involvement of residue Asn-273 in the coordination of an active site water molecule suggests that this C-terminal structural motif may play a role in the substrate recognition.

**Mechanisms for the Dual Substrate Specificity of hsNMNAT and Its Implications**—The more relaxed substrate specificity of human NMNAT contrasts with the stringent substrate preference of bacterial and archaeal NMNATs. *E. coli* and many other bacterial NMNATs strongly prefer the deamidated NMN to NMN, with the ration of the apparent second order constant $k_{cat}/K_m$ for the two substrates exceeding 10$^3$-fold (25, 26), whereas archaeal NMNAT prefers the amidated NMN to NaMN by about 2 orders of magnitude (27). The structural comparisons of NMNATs from human, *E. coli*, and archaeal species and their complexes with respective ligands (21, 23, 24) enable us to deduce the structural mechanisms underlining the different substrate specificities. In *E. coli* NMNAT, an anion-binding pocket created by the main chain amides of Leu-117, Tyr-85, Ala-86, and the positive dipole of helix C is responsible for the recognition of the negatively charged carboxylate group of NaMN. The electrophilic nature of this pocket is further enhanced by the surrounding hydrophobic residues, which would increase its ability to discriminate between a carboxylate and a carboxamide. In archaeal NMNAT, the pyridine-binding site is substantially different from those in *E. coli* and human enzymes, and the bound pyridine nucleotide adopts a rather different conformation than those in *E. coli* and human NMNAT-ligand complexes (21). The specific interactions with the nicotinamide include hydrogen bonding between both amide and carbonyl groups of the Ile-81 main chain and the carbamino groups of NMN (24). These interactions clearly are more favorable for the binding of a carboxamide than a carboxylate.

Compared with the bacterial and archaeal NMNAT active sites, one prominent feature of the human NMNAT pyridine-binding site is the presence of several conserved water molecules ($\omega 1$–$4$) and a negatively charged Asp residue (Asp-173). Based on the structural comparison of hsNMNAT complexed with NAD or NaAD, we propose that human NMNAT recognizes both NMN and NaMN readily without the need for any significant conformational changes. The presence of Asp-173 and several conserved water molecules could change the subtle electrostatic distributions of the binding site and enable the enzyme to accommodate substrates of different electrostatic properties. This intrinsically relaxed substrate specificity makes human NMNAT flexible in balancing different NMN/NaMN metabolic fluxes for NAD generation, which may be required to satisfy the requirement for NAD in nuclei.

The distinct substrate specificity patterns of NMNATs from different organisms are indicative of significant variations in the metabolite fluxes in the cell that lead to the NAD production. The unique dual specificity evolved by hsNMNAT has
clear biological implications with respect to possible NAD biosynthetic pathways in human, which include both types of intermediates, NaMN and NMN (Fig. 1). The relative importance of these pathways in different types of tissues and in different physiological states remains to be studied. In the majority of bacterial pathogens, such as Staphylococcus aureus, Streptococcus pneumoniae, and Helicobacter pylori, the preferred intermediate in NAD biosynthesis is NaMN. It correlates with a strong preference of bacterial NMNAT (NadD family) toward this form of pyridine mononucleotide (25). Significant differences between bacterial and human enzymes in both substrate specificity and the active site conformations, as revealed in this study, are of crucial importance for the design of highly selective NMNAT inhibitors with anti-infectious potential.

Interactions with β-CH₂-TAD—To understand the mechanism of NMNAT-catalyzed adenylation of the oncolytic agent tiazofurin nucleotide, we solved the structure of human NMNAT complexed with a non-cleavable tiazofurin adenine dinucleotide analogue, β-CH₂-TAD (Fig. 5). β-CH₂-TAD is structurally similar to TAD (Fig. 1b) but is resistant to the reverse pyrophosphorylation reaction catalyzed by NMNAT (16). The active site of the enzyme appears to accommodate β-CH₂-TAD easily; the bulkier sulfur atom on the thiazole ring is cis to the furanose oxygen O1 and is pointed toward the solvent. The distance between the sulfur and O1 is 3.16 Å, slightly longer than that in the free tiazofurin (2.958 Å) (28). This sulfur atom is also adjacent to the hydroxyl of Tyr-55 side chain, with the S-O distance (3.7 Å) close to the sum of S and O van der Waals radii (3.57 Å) (29). The nitrogen atom on the thiazole ring is found to form a hydrogen bond with the Thr-95 side chain hydroxyl (2.7 Å) (Fig. 5b). The glycosyl torsion angle χ(01—C1—C2—S) of bound β-CH₂-TAD is about 50°, slightly larger than that of NAD (–36°) and free tiazofurin (31°) (28) but very similar to that of ara-tiazofurin (55°) (30). Similar to the free tiazofurin, the furanose moiety of the bound β-CH₂-TAD also adopts a C2'-endo pucker. However, in contrast to the free tiazofurin in which the carbamoyl nitrogen is cis to the thiazole nitrogen (28), the carbamoyl moiety of the enzyme-bound β-CH₂-TAD is oriented with the NH₂ group trans to the ring nitrogen. Clearly, interactions with the surrounding protein atoms largely dictate the orientation of the carbamoyl. As a result, the conformation of the bound β-CH₂-TAD is very similar to that of bound NAD, and they form essentially the same interactions with the enzyme, except for the extra interactions with the sulfur and nitrogen atoms on the thiazole ring (Fig. 5b). The NaMNAT active site would also be able to accommodate the tiazofurin analogue selenozofurin, which has been shown to be more cytotoxic to tumor cells in vitro than tiazofurin (31).

One of the problems associated with tiazofurin chemotherapy is the development of drug resistance (15). The most prominent difference between the resistant cells and the sensitive cells is that NMNAT activities are drastically depleted, resulting in the accumulation of phosphorylated tiazofurin and a reduction of the active TAD pool (32, 33). This could be due to the direct suppression of the enzyme by TAD, enhanced reverse reaction rate, or indirect alteration of the genes or proteins that regulate NMNAT expression and activity. Elucidating the interactions between tiazofurin nucleotide and NMNAT represents a step forward in understanding the mechanisms of the metabolic activation of tiazofurin and related nucleoside prodrugs. Further biochemical studies of NMNAT kinetic properties and the regulation of NAD metabolism as a whole are required to produce strategies for preventing or overcoming the development of resistance to tiazofurin.

In summary, our structural data suggest that the conformation of the tiazofurin moiety of the NMNAT-bound TAD is very similar to that of the free tiazofurin. Therefore, human NMNAT would recognize tiazofurin nucleotide readily and hence catalyze its adenylation to generate TAD. The detailed structural knowledge of the active site of hαNMNAT is valuable for the design and improvement of tiazofurin-related nucleoside anticancer drugs, because their efficient conversion by cellular enzymes, including nucleoside kinase and especially NMNAT, is crucial for their antitumor activity.

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