Capture of a Labile Substrate by Expulsion of Water Molecules from the Active Site of Nicotinate Mononucleotide: 5,6-Dimethylbenzimidazole Phosphoribosyltransferase (CobT) from Salmonella enterica*

Received for publication, April 12, 2002, and in revised form, June 24, 2002
Published, JBC Papers in Press, July 5, 2002, DOI 10.1074/jbc.M203535200

Cheom-Gil Cheong†, Jorge C. Escalante-Semerena§, and Ivan Rayment¶

From the Departments of Biochemistry and Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Nicotinate mononucleotide (NaMN): 5,6-dimethylbenzimidazole (DMB) phosphoribosyltransferase (CobT) from Salmonella enterica plays a central role in the synthesis of α-ribose-5′-phosphate, an intermediate for the lower ligand of cobalamin. In earlier studies it proved difficult to obtain the structure of CobT bound to NaMN because it is hydrolyzed in the crystal lattice in the absence of the second substrate DMB. In an effort to map the reaction pathway of this enzyme, NaMN was captured in the active site with the substrate analogs map the reaction pathway of this enzyme, NaMN was captured in the active site with the substrate analogs 4,5-dimethyl-1,2-phenylenediamine, 4-methylcatechol, indole, 3,4-dimethylaniline, 2,5-dimethylaniline, 3,4-dimethylphenol, and 2-amino-p-cresol. Structures of these complexes reveal that they exclude water molecules responsible for the hydrolysis from the active site. These structures, together with the early complexes accompanied by a disorder-order transition in a surface loop. The structure of apo-CobT is also reported.

Cobalamin is the largest and most complex cofactor found in biological systems (Fig. 1). It contains a cobalt ion at the center of a corrin ring that contributes four nitrogen atoms to the coordination sphere of the cobalt ion. Like other macrocyclic tetrapyrroles, the corrin ring has a conjugated double bond system that gives its unique red color. Unlike other macrocyclic tetrapyrroles, the coordination sphere is completed by an upper ligand. These cobamides are called adenosylcobalamin, methycobalamin, and cyanocobalamin, respectively. The upper ligand is attached to the cobalt ion by the unusual and labile metal-carbon covalent bond. The lower ligand of the cobamide synthesized by Salmonella enterica under aerobic growth conditions is 5,6-dimethylbenzimidazole, but under anaerobic growth conditions this bacterium synthesizes pseudo-B12 or Co(α)adenylcobamide (1, 2). A wide variation of the lower ligand among cobamide-producing procaryotes has been reported (3). This variability is of interest because cobalamin is utilized by a wide range of higher organisms regardless of the nature of the lower ligand.

The biosynthesis of cobalamin has attracted much interest because of its complexity, but also because it arose early in the evolution of life where it was synthesized only by Archaea and some bacteria. To date, 24 genes have been identified as involved in the biosynthesis of this cofactor (4). Of these, four enzymes, CobU, CobT, CobC, and CobS, in S. enterica are involved in the synthesis of the lower ligand and the assembly of the nucleotide loop (Fig. 2) (5). This process starts with guanylation of the 1-amino-2-propanol-phosphate side chain of adenosylcobinamide phosphate by CobU. CobU is a bifunctional enzyme and has adenosylcobinamide kinase/adenosylcobinamide phosphate guanylyltransferase activities (6, 7). Thereafter, the GMP moiety of the activated adenosylcobinamide-GDP is displaced by α-ribose in the reaction catalyzed by CobS, cobalamin synthase, to form the final product, adenosylcobalamin (8, 9). α-Ribose is synthesized through the sequential action of CobT and CobC (Fig. 2), where CobT is a phosphoribosyltransferase (10) and CobC is a phosphatase (11). CobT has a molecular weight of 39,000 (10) and catalyzes the transfer of the phosphoribosyl group from NaMN to 5,6-dimethylbenzimidazole (DMB)1 to form α-ribose-5′-phosphate (Scheme 1). This latter enzyme is unusual in that it utilizes nicotinate mononucleotide (NaMN) as the phosphoribosyl group donor rather than the more abundant NMN where the Kₘ values for NaMN and NMN are 0.68 and 30 mM, respectively (10). CobT is also interesting because it synthesizes α-nucleotides via a phosphoribosyltransferase reaction utilizing NaMN as the phosphoribosyl donor in contrast to the much larger family of phosphoribosylpyrophosphate-dependent transferases that synthesize β-nucleotides (12–14).

* This research was supported in part by National Institutes of Health Grants GM58281 (to I. R.) and GM40313 (to J. E-S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1L4B, 1L4F, 1L4G, 1L4H, 1L4K, 1L4L, 1L5O, 1L4M) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† To whom correspondence may be addressed: Dept. of Biochemistry, 433 Babcock Dr., Madison, WI 53706. Tel.: 608-262-0437; Fax: 608-262-1319; E-mail: Ivan_Rayment@biochem.wisc.edu.

‡ To whom correspondence may be addressed: Dept. of Bacteriology, Fred Hall, Linden Dr., Madison, WI 53706. E-mail: Escalante@bact.wisc.edu.

¶ The abbreviations used are: DMB, 5,6-dimethylbenzimidazole; NaMN, nicotinate mononucleotide; NMN, nicotinamide mononucleotide; DMPDA, 4,5-dimethyl-1,2-phenylenediamine; 3,4-DMP, 3,4-dimethylphenol; r.m.s., root mean square; RCSB, Research Collaboratory for Structural Bioinformatics.

1 The abbreviations used are: DMB, 5,6-dimethylbenzimidazole; NaMN, nicotinate mononucleotide; NMN, nicotinamide mononucleotide; DMPDA, 4,5-dimethyl-1,2-phenylenediamine; 3,4-DMP, 3,4-dimethylphenol; r.m.s., root mean square; RCSB, Research Collaboratory for Structural Bioinformatics.

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

This paper is available on line at http://www.jbc.org
The three-dimensional structure of CobT from *S. enterica* has been determined complexed with its substrate, DMB, and also with its reaction products, nicotinate and α-ribazole-5'-phosphate (15). These studies revealed that CobT is a molecular dimer, and each subunit is composed of a large and a small domain (Fig. 3). The large domain exhibits a Rossmann fold, which is characterized by a six-stranded parallel β-sheet and found in dinucleotide binding proteins. The active site is located at the C-terminal end of the β-sheets that form the core of the Rossmann fold. The active site is built from the loops that emerge from the Rossmann fold together with components of the small domain, where the DMB binding pocket is formed by the hydrophobic residues provided by both subunits of the dimer. Interestingly, the orientation of the nucleotide across the β-sheet is opposite to that normally observed in other dinucleotide binding proteins (15).

As noted earlier, cobamide contains a wide range of aromatic bases as the lower ligand to the cobalt, which varies according to the biosynthetic organism (16). The diversity of lower ligands appears to be due to a lack of specificity in the phosphorylribosyl transferase because CobT from *S. enterica* has been shown to utilize benzimidazole, imidazole, histidine, DMPDA, and guanine in biochemical assays (10). Structural studies of CobT complexed with the alternative lower ligand bases such as adenine, 5-methylbenzimidazole, 5-methoxybenzimidazole, p-cresol, and phenol revealed that CobT can accommodate all of the commonly observed aromatic bases that constitute the lower ligand (17). Furthermore, except for the phenolic bases, the phosphorylribosyl transfer reaction occurs readily within the crystalline lattice to yield a product with the correct stereochemistry, as observed in the natural transfer reactions where the stereospecificity is determined by Ser-80 and Gln-88, located at the periphery of the hydrophobic pocket (17).

**MATERIALS AND METHODS**

**Protein Purification**—CobT was overexpressed and purified as described before (10, 15). All purification procedures and manipulations of
the protein were carried out at 4 °C. The purified protein was concentrated in a Centriprep-30 and dialyzed against 20 mM Tris-HCl, pH 7.5, containing 100 mM NaCl. The protein was flash-frozen by dropping 32 μl of aliquots into liquid nitrogen that were then stored at −80 °C.

Crystallization and X-ray Data Collection—Crystals of apo-CobT employed for this structural investigation were grown with the hanging drop vapor diffusion technique. Equal volumes of protein at 6 mg/ml protein in its final storage buffer and a precipitant containing 1.4M NH₄H₂PO₄/(NH₄)₂HPO₄, at pH 6.0, were mixed and suspended over the precipitant solution at room temperature. Crystals grew spontaneously and achieved sizes of 0.6 mm × 0.6 mm × 0.2 mm in 2 weeks. The crystals belong to the space group P2₁2₁2₁ with unit cell dimensions of a = 72.1 Å, b = 90.2 Å, and c = 47.5 Å.

Prior to preparation of the ligand complexes, the crystals of apo-CobT were transferred to 1.4 M NH₄H₂PO₄/NH₄₂HPO₄, pH 6.0, in which they were indefinitely stable. All the substrate analogs and NaMN were dissolved in this storage solution. Crystals were first transferred to a solution of the analogs; after 24 h or more, a 100 mM NaMN solution was added to a final concentration of 10 mM. The concentrations of the lower ligands and the length of the soak are listed in Table II.

X-ray data were collected at 5 °C with a Siemens HiStar area detector at a crystal-to-detector distance of 12 cm. CuKα radiation was generated by a Rigaku RU200 x-ray generator operated at 50 kV and 90 mA and equipped with a set of double focusing mirrors (Charles Supper Co). Diffraction data frames, 0.15° wide, were recorded for 60 or 90 s. The frames were processed with XDS (18, 19) and internally scaled with

### Table I

| Aromatic Bases                  | Structures | Aromatic Bases                  | Structures |
|---------------------------------|------------|---------------------------------|------------|
| 5,6-Dimethyl-benzimidazole      | ![](image1) | 3,4-Dimethylaniline             | ![](image2) |
| (DMB)                           | ![](image3) | (3,4-DMAN)                      | ![](image4) |
| 4,5-Dimethyl-1,2-phenylenediamine| ![](image5) | 2,5-Dimethylaniline             | ![](image6) |
| (DMPDA)                         | ![](image7) | (2,5-DMA)                       | ![](image8) |
| 4-Methylcatechole                | ![](image9) | 3,4-Dimethylphenol              | ![](image10) |
|                                 | ![](image11) | (3,4-DMP)                       | ![](image12) |
| Indole                          | ![](image13) | 2-Amino-p-creols               | ![](image14) |

* Reported previously by Cheong et al. (15).
* Solved and discussed in the present study.
* Deposited in the RCSB data bank.
The starting model for the structural determinations was obtained by omitting DMB and water molecules from the CobT/DMB complex (RCSB accession number 1D0S) (15). This initial model was refined with the programs TNT or CNS against each data set (20, 21). The overall structure of apo-CobT is very similar to that of the previous complexes (15, 17), in which the overall r.m.s. difference between the positions of the ordered α-carbons in the CobT-DMB complex and apo-CobT structures is 0.17 Å. The only significant difference occurs in the region surrounding the aromatic base where the loop that extends from residue Met-344 to Gly-349 is disordered in the absence of an aromatic base. This loop provides the hydrophobic residues that interact with DMB and appears to close off the binding pocket. It is also the section of the binding pocket that is contributed by the neighboring 2-fold related subunit (15). This suggests that CobT undergoes a localized conformational change when it binds the substrate DMB. Indeed, without this conformational change it would be difficult to insert DMB into the binding pocket. Initial attempts to capture the substrate NaMN in the active site by soaking in 50 mM NaMN solution in 1.4 M ammonium phosphate, pH 6.0, for 1 day were unsuccessful. When data were recorded from such crystals, the difference Fourier maps showed only nicotinate and a phosphate in the active site. This suggests that CobT hydrolyzes NaMN in the absence of the second substrate and that the ribose-5-phosphate is displaced by phosphate that exists at high concentration in the soaking solution. Such a side reaction would be physiologically wasteful and might be ameliorated by either ordered binding of substrates or by normal saturation of the enzyme with the aromatic base. Consideration of the $K_m$ for the two substrates

### Table II

| Apo<sup>a</sup> | DMPDA NaMN<sup>b</sup> | 4-Methylcatechol NaMN<sup>b</sup> | Indole NaMN<sup>b</sup> |
|-----------------|------------------|-------------------|--------------------|
| Conc. (mm)      | 1                | 1                 | 1                  |
| Length of soak (days) | 10               | 10                | 10                 |
| Number of crystals | 1               | 1                 | 1                  |
| Resolution (Å)  | 1.7              | 2.1               | 2.1                |
| Average Io      | 13.8 (1.6)       | 10.8 (3.1)        | 11.2 (3.3)         |
| Unique reflections | 31865            | 15485             | 15491              |
| Redundancy      | 3.3 (1.3)        | 2.0 (1.1)         | 1.9 (1.1)          |
| Completeness (%)| 88.4 (60.6)      | 93.9 (85.4)       | 89.0 (68.3)        |
| $R_{merge}$ (%) | 6.3 (27.9)       | 4.7 (13.4)        | 4.3 (12.2)         |

<sup>a</sup> The values in parentheses are from resolution shells of 1.75–1.70 Å.

<sup>b</sup> The values in parentheses are from resolution shells of 2.16–2.1 Å.

<sup>c</sup> $R_{merge} = \Sigma |I_i| - |I_i|/\Sigma |I_i|$, where $I_i$ and $I_o$ are the intensities of individual and mean structure factors.

### Table III

| Apo<sup>a</sup> | DMPDA NaMN<sup>b</sup> | 4-Methylcatechol NaMN<sup>b</sup> | Indole NaMN<sup>b</sup> |
|-----------------|------------------|-------------------|--------------------|
| Resolution limits (Å) | 1.7              | 2.1               | 2.1                |
| Final $R_{factor}$ (%) | 19.1             | 18.1              | 18.6               |
| $R_{free}$ (%)   | 21.8             | 22.1              | 22.6               |
| No. of reflections (working set) | 29285            | 14335             | 14359              |
| No. of reflections (test set) | 1541            | 758               | 755                |
| No. of protein atoms | 2362            | 2428              | 2431               |
| No. of solvent molecules | 119           | 94               | 120                |
| Other molecules, ions | 1 PO4           | DMPDA             | 4-Methylcatechol    |
| Average B values Å<sup>2</sup> | -Main chain atoms | 20.4             | 19.0               |
|                   | -All protein atoms | 22.2             | 20.3               |
|                   | -Solvent atoms    | 33.2             | 29.6               |
|                   | -Ligand atoms     | 25.8             | 14.4               |
| Weighted r.m.s. deviations from ideality | Bond lengths (Å) | 0.009           | 0.006              |
|                   | Bond angles (deg) | 1.34             | 1.17               |
|                   | r.m.s. coordinate error (Å)<sup>c</sup> | 0.20           | 0.22               |
| RCSB accession number | 1L4B            | 14LF             | 1L4G               |

<sup>a</sup> Refinement with CNS.

<sup>b</sup> $R_{factor} = \Sigma |F_o| - |kF_c|/\Sigma |F_o|$, $R_{free}^R_{factor}$ for 5% of the data excluded from the refinement.

<sup>c</sup> Coordinate errors are those calculated through a Luzzati analysis.

### RESULTS AND DISCUSSION

**Apo-CobT**—The overall structure of apo-CobT is very similar to that of the previous complexes (15, 17), in which the overall r.m.s. difference between the positions of the ordered α-carbons in the CobT-DMB complex and apo-CobT structures is 0.17 Å. The only significant difference occurs in the region surrounding the aromatic base where the loop that extends from residue Met-344 to Gly-349 is disordered in the absence of an aromatic base. This loop provides the hydrophobic residues that interact with DMB and appears to close off the binding pocket. It is also the section of the binding pocket that is contributed by the neighboring 2-fold related subunit (15). This suggests that CobT undergoes a localized conformational change when it binds the substrate DMB. Indeed, without this conformational change it would be difficult to insert DMB into the binding pocket.

Initial attempts to capture the substrate NaMN in the active site by soaking in 50 mM NaMN solution in 1.4 M ammonium phosphate, pH 6.0, for 1 day were unsuccessful. When data were recorded from such crystals, the difference Fourier maps showed only nicotinate and a phosphate in the active site. This suggests that CobT hydrolyzes NaMN in the absence of the second substrate and that the ribose-5-phosphate is displaced by phosphate that exists at high concentration in the soaking solution. Such a side reaction would be physiologically wasteful and might be ameliorated by either ordered binding of substrates or by normal saturation of the enzyme with the aromatic base. Consideration of the $K_m$ for the two substrates
suggests that the latter might be true because the $K_m$ for NaMN is considerably higher than that of DMB ($K_m$ is 680 μM for NaMN and less than 10 μM for DMB) (10). This suggests that under normal circumstances the enzyme would be complexed with an aromatic base, which should reduce hydrolysis of NaMN.

**NaMN Complexes**—The structure of NaMN bound to CobT was captured by first soaking the crystals in an inactive analog of the commonly utilized aromatic bases and then transferring those crystals to a solution that contained 10 mM NaMN. A series of compounds was examined with the goal of understanding the features of the substrate and active site necessary for catalysis. This list included 4,5-dimethyl-1,2-phenylenediamine, 4-methylcatechol, indole, 3,4-dimethylaniline, 2,5-dimethylaniline, 3,4-dimethylphenol, and 2-amino-p-cresol (Table I). The difference Fourier maps for all of these complexes showed unhydrolyzed NaMN in the active site in conjunction with the aromatic analog. For brevity the data statistics and electron density for the 4,5-dimethyl-1,2-phenylenediamine, 4-methylcatechol, and indole NaMN complexes are shown in Table II and III and Fig. 4 where the others have been deposited in the RCSB (accession numbers 1L4K, 1L4L, 1L5O, and 1L4M for 3,4-dimethylaniline, 2,5-dimethylaniline, 3,4-dimethylphenol, and 2-amino-p-cresol, respectively). In binding to CobT, these aromatic compounds occupy the space adjacent to the ribose moiety and exclude water molecules from the proximity of C1’ of the phosphoribosyl moiety, thus preventing hydrolysis of NaMN.

**Location of the Aromatic Analogs**—Comparison of the chemical structures of 4,5-dimethyl-1,2-phenylenediamine, 4-methylcatechol, and indole (Table I) reveals that the only structural
feature they share is a benzene ring. Otherwise they contain different hydrogen bonding substitutions and numbers of methyl groups attached to the benzene ring opposite the polar constituents. Even so, these compounds adopt very similar locations in the active site (Fig. 5A). The one feature that appears to control the overall position of these DMB analogs is the universal hydrogen bond between Glu-317 and either the hydroxyl, amino group, or ring nitrogen of indole. In the latter compound there is only one hydrogen bond donor in contrast to the other two compounds, so that the orientation is chosen to maintain the hydrogen bond. These compounds emphasize the importance of Glu-317 and suggest that one of its roles is to orient the organic bases in the active site opposite to the C1’ of the ribose moiety.

Each of the three analogs presents a different type of atom to the C1’ of the ribose. In the case of 4,5-dimethyl-1,2-phenylenediamine, the amino group hydrogen bonds to the ring oxygen and O2’ of the ribose with distances of 2.7 and 3.2 Å, respectively, and lies within 2.9 Å of the C1’. The geometry of this interaction would be appropriate for nucleophilic attack on C1’; however, the reaction does not appear to occur even though the amino group adjacent to C1’ is expected to be deprotonated (pKₐ ~ 4.5). The most likely explanation for this is the absence of a catalytic base in the appropriate location for abstraction of the ribose. For 4-methylcatechol, the second hydroxyl group forms a hydrogen bond to the carbonyl oxygen of Leu-315 that is 3.0 Å long and lies within 3.1 and 2.8 Å of the O2’ and O3’ hydroxyl groups of ribose, respectively. The hydroxyl also lies 3.0 Å from C1’ of the ribose and, in principle, should be poised for nucleophilic attack on the ribose. However, in the absence of a strong base, that would be unlikely to occur because of the relatively high pKₐ of the hydroxyl group (~9). It is noteworthy that for 4-methylcatechol, the methyl group is pointing to the 5-methyl binding pocket of the DMB-binding site, which as noted previously is a determinant for the orientation of bases within the active site (17). The fact that the hydroxyl or amino group of 4-methylcatechol or DMPDA cannot react with NaMN also supports that Glu-317 is the catalytic base in the active site for the normal reaction. Finally, for indole the C3 of the analog lies ~4.5 Å from C1’ of the ribose. As expected, this atom does not make favorable interactions with any components of NaMN and lies beyond the van der Waals distance for these atoms.

**Location of NaMN**—When all the structures of the unhydrolyzed NaMN complexes are superimposed, NaMN occupies the same site while small variations of the binding of the substrate analogs are observed (Fig. 5A). The binding mode of the phosphate and nicotinate part of NaMN is very similar to that in the α-ribazole-5’-phosphate complex (Fig. 5B). All hydrogen bonds are the same except for small differences in hydrogen bond distances.

The hydrogen bonding pattern surrounding the ribose moiety in both NaMN and α-ribazole-5’-phosphate is also very similar, considering its overall movement of ~1 Å from substrate to product. Indeed, there are relatively few interactions between the ribose and the protein in either complex. The most prominent hydrogen bonding contacts are first, between O2’ and Glu-174 Oe with a distance of 2.8 and 2.6 Å in substrate and products complex, respectively, and second, between O3’ and the amide hydrogen of Gly-176 with distances of 3.1 and 2.9 Å. There is one additional interaction seen in the substrate complex between the carbonyl oxygen of Leu-315 and O2’ with a distance of 3.0 Å, where the corresponding distance in the products complex is 3.7 Å. The first two sets of interactions are conserved, because they lie approximately in the plane of the ribose ring and are less affected by the movement of the ring,
which occurs along a vector approximately perpendicular to this plane. In hindsight, the presence of few interactions to the ribose is appropriate considering that this part of the molecule must move for the reaction to occur. A tight hydrogen bonding pattern in either the substrate or product state would be deleterious for catalysis. Increased interactions in the substrate would impede progress toward the transition state, whereas stronger binding of the product (α-ribazole-5′-phosphate) would be expected to reduce the affinity for the substrate and consequently increase the $K_m$ for the NaMN above its already comparatively high value.

The structures of CobT complexed with unhydrolyzed NaMN, together with the previously determined structures of CobT-DMB and CobT-products complexes, provide snapshots along the reaction pathway. Superimposition of the structures of the DMB complex, unhydrolyzed NaMN complexes, and α-ribazole-5′-phosphate complex show that nicotinate and phosphate moieties do not move during the reaction but suggest that the ribose and DMB moieties move significantly toward each other (Figs. 5B and 6A). As estimated from the superimposition of the 4,5-dimethyl-1,2-phenylenediamine and NaMN complex and the DMB complex and α-ribazole-5′-phosphate complex, the C1′ atom of ribose ring moves 1.5 Å and the N1 atom of DMB moves 0.9 Å during the reaction (Fig. 6). When superimposed, the distances between the N1 atom of DMB in the DMB complex and the C1′ atom of ribose of NaMN in seven unhydrolyzed NaMN complexes are ~3.6 Å apart (van der Waals contact distance) and are oriented appropriately for reaction to occur. Significantly, the amino acid residues involved in substrate binding do not appear to move significantly during the reaction.

The structures presented here support the catalytic mechanism set forward in Fig. 6. For the normal substrates utilized by CobT it is expected that the imidazole moiety will be neutral at a pH of ~7 and carry a single proton on one of the ring nitrogens. Thus the initial role of Glu-317 is suggested to be orientation of the imidazole moiety in the active site with the protonated nitrogen directed toward this hydrogen bonding acceptor. As a consequence, this places the unprotonated nitrogen opposite the C1′ atom of the ribose in preparation for an in-line displacement reaction. For reaction to occur, these two groups must approach one another where it would appear that the lâx hydrogen bonding pattern associated with the ribose favors this transition.

CONCLUSIONS

This study completes the structural characterization of the key steps in the nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase reaction catalyzed by CobT. This was made possible through utilization of a substrate analog of the aromatic base to trap the hydrolytically unstable NaMN in the active site. Clearly the analogs serve to exclude water from the active site adjacent to C1′ of the ribose, even though no ordered water molecules are observed within the binding pockets of either the analog complexes or apo structure. This suggests that the water molecules responsible for hydrolysis of NaMN are transitory in nature. The hydrolysis of the substrate NaMN in the absence of the other aromatic substrate suggests that CobT must bind DMB first to avoid the wasteful hydrolysis of the nucleotide, where this seems to be achieved by the big difference of $K_m$ values between two substrates.

The structure of apo-CobT showed that disorder/order transition occurs in the C-terminal loop when DMB binds to CobT. This C-terminal loop provides several hydrophobic residues that are involved in substrate specificity. Conformational flexibility in this part of the molecule is likely necessary to allow the substrates to enter the active site.

One of the purposes for undertaking this study was to determine the reason for the specificity of CobT for NaMN over the more abundant NMN, where there is a 44-fold lower $K_m$ for the former substrate over the latter. As shown here, there is little difference in the position of the nicotinate group in the putative substrate complex and the products complex. Furthermore, there are few interactions between the carboxylate group and the protein, so it is difficult to identify a specific reason for the lower affinity for NMN. This question must await further study.

Acknowledgment—we thank Dr. Hazel M. Holden for helpful discussions.

REFERENCES

1. Keck, B., Munder, M., and Renz, P. (1996) Arch. Microbiol. 171, 66–68
2. Keck, B., and Renz, P. (2000) Arch. Microbiol. 173, 76–77
3. Rondon, M. R., Trzebiatowski, J. R., and Escalante-Semerena, J. C. (1997) Prog. Nucleic Acid Res. Mol. Biol. 56, 347–384
4. Roth, J. R., Lawrence, J. G., and Bobik, T. A. (1996) *Annu. Rev. Microbiol.* **50**, 137–181
5. Maggio-Hall, L. A., and Escalante-Semerena, J. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 11798–11803
6. O’Toole, G. A. (1994) *Biochemistry and Genetics of Cobalamin Nucleotide Loop Assembly in Salmonella typhimurium*. Ph.D. thesis, University of Wisconsin, Madison
7. O’Toole, G. A., and Escalante-Semerena, J. C. (1995) *J. Biol. Chem.* **270**, 23560–23569
8. O’Toole, G. A., Rondon, M. R., and Escalante-Semerena, J. C. (1993) *J. Bacteriol.* **175**, 3317–3326
9. Roth, J. R., Lawrence, J. G., Rubenfield, M., Kieffer-Higgins, S., and Church, G. M. (1993) *J. Bacteriol.* **175**, 3303–3316
10. Trzebiatowski, J. R., and Escalante-Semerena, J. C. (1997) *J. Biol. Chem.* **272**, 17662–17667
11. O’Toole, G. A., Trzebiatowski, J. R., and Escalante-Semerena, J. C. (1994) *J. Biol. Chem.* **269**, 26503–26511
12. Musik, W. D., and Nyahn, W. L. (1981) *CRC Crit. Rev. Biochem.*
13. Craig, S. P., III, and Eakin, A. E. (2000) *J. Biol. Chem.* **275**, 20231–20234
14. Grubmeyer, C. T., Gross, J. W., and Rajavel, M. (1999) *Methods Enzymol.* **308**, 28–48
15. Cheong, C. G., Escalante-Semerena, J. C., and Rayment, I. (1999) *Biochemistry* **38**, 16125–16135
16. Perlman, D., and Barrett, J. M. (1958) *Can. J. Microbiol.* **4**, 9–15
17. Cheong, C. G., Escalante-Semerena, J. C., and Rayment, I. (2001) *J. Biol. Chem.* **276**, 37612–37620
18. Kabsch, W. (1988) *J. Appl. Crystallogr.* **21**, 67–71
19. Kabsch, W. (1988) *J. Appl. Crystallogr.* **21**, 916–924
20. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, I. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D* **54**, 905–921
21. Tronrud, D. E. (1997) *Methods Enzymol.* **277**, 306–319
22. Luzzati, V. (1952) *Acta Crystallogr.* **5**, 802–810
23. Esnouf, R. M. (1999) *Acta Crystallogr. Sect. D* **55**, 938–940
24. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950