Unusual folded conformation of nicotinamide adenine dinucleotide bound to flavin reductase P

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Abstract

The 2.1 Å resolution crystal structure of flavin reductase P with the inhibitor nicotinamide adenine dinucleotide (NAD) bound in the active site has been determined. NAD adopts a novel, folded conformation in which the nicotinamide and adenine rings stack in parallel with an inter-ring distance of 3.6 Å. The pyrophosphate binds next to the flavin cofactor isoalloxazine, while the stacked nicotinamide/adenine moiety faces away from the flavin. The observed NAD conformation is quite different from the extended conformations observed in other enzyme/NAD(P) structures; however, it resembles the conformation proposed for NAD in solution. The flavin reductase P/NAD structure provides new information about the conformational diversity of NAD, which is important for understanding catalysis. This structure offers the first crystallographic evidence of a folded NAD with ring stacking, and it is the first enzyme structure containing an FMN cofactor interacting with NAD(P). Analysis of the structure suggests a possible dynamic mechanism underlying NADPH substrate specificity and product release that involves unfolding and folding of NADP(H).

Keywords: flavin reductase P (FRP); folded NAD; NAD conformation; NAD(P)H:FMN oxidoreductase; NAD(P)H:flavin oxidoreductase; NADH oxidase/flavine reductase nicotinamide adenine dinucleotide (NAD)

Nicotinamide adenine dinucleotides (NAD, NADP) are substrates and coenzymes in many biochemical reactions, including electron transfer and ADP-ribosylation (Jacobson & Jacobson, 1989). The reactive functionality in electron transfer reactions is the nicotinamide, with hydride transfer occurring at the C4 atom. The rest of the molecule (two ribose rings, pyrophosphate, adenine, and adenosine 2'-phosphate in the case of NADP) is responsible for specificity and affinity of enzyme binding. Two conformational families of NAD(P) exist: extended molecules bound to enzymes and folded NAD(P) molecules free in solution. The extended form has been well documented by crystallography of enzyme/NAD(P) complexes, while little detailed structural information is available for the folded form.

This paper reports the first crystal structure of a folded NAD bound to an enzyme. Our focus is flavin reductase P from *Vibrio harveyi* (FRP), which is an NAD(P)H:flavin oxidoreductase. Flavin reductases catalyze the reduction of flavins by NAD(P)H (Equa-

tion 1). The reduced flavin product is an important unit of currency in biochemical redox reactions, and it is essential to many biological functions, including iron release from ferrisiderophores (Hallé & Meyer, 1992; Covès & Fontecave, 1993), activation of ribonucleotide reductase (Fontecave et al., 1987; Covès et al., 1993) and chorismate synthase (Hasan & Nestor, 1978), reduction of methemoglobin (Quandt et al., 1991; Chikuba et al., 1994), oxygen activation (Gaudu et al., 1994), biosynthesis of the antitumor agent valanimycin (Parry & Li, 1997), and desulfurization of fossil fuel (Lei & Tu, 1996). Much of the interest in flavin reductases concerns elucidation of their role in bacterial bioluminescence (Tu & Mager, 1995). It is thought that flavin reductases provide reduced FMN (FMNH₂) to luciferase in vivo as a substrate for the luminescence reaction (Equations 1, 2).

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Abbreviations: ADP, adenosine diphosphate; FRase I, the major NAD(P)H:FMN oxidoreductase of *Vibrio fischeri*; FRP, flavin reductase P from *Vibrio harveyi*; NAD, nicotinamide adenine dinucleotide; NfsA, major *E. coli* NADPH-dependent nitroreductase; NfsB, a minor *E. coli* nitroreductase; NOX, NADH oxidase from *Thermus thermophilus*.

Several flavin reductases have been isolated, and they have a wide range of specificities regarding their flavin cofactor, flavin

substrates, and dinucleotide substrates. FRP contains an FMN co-factor, and it reduces the flavin substrates FMN and riboflavin (Lei et al., 1994; Lei & Tu, 1994; Liu et al., 1997). NADPH is the preferred source of reducing electrons for FRP. NADH is a poor substrate, while NAD inhibits the activity of FRP.

We previously determined the crystal structure of FRP at 1.8 Å resolution (Tanner et al., 1996b), which allowed us to characterize the overall fold, locate the active site, and describe the protein-co-factor interactions. The FRP/NAD complex provides new information about NAD conformation, and it offers insights about the structural basis of inhibition of FRP by NAD and perhaps about the NADPH specificity of FRP.

Results

Quality of the structure

The refined FRP/NAD structure contains 460 amino acid residues, two FMN molecules, one NAD molecule, and 73 water molecules, with $R_{\text{test}} = 20.6\%$ and $R_{\text{work}} = 18.5\%$. The root-mean-square deviation (RMSD) from the ideal force field values is 0.005 Å for bonds, 1.3° for angles, 22.7° for dihedral angles, and 1.4° for improper dihedrals. The RMS difference between the native and NAD-complexed structures after least-squares superposition of the CA atoms is 0.10 Å for CA atoms and 0.15 Å for all atoms.

The average B -factor is 12.5 Å² for protein, 8.7 Å² for FMN, 13.0 Å² for water, and 26.2 Å² for NAD. The highest B -factor is 46 Å² and occurs in Tyr B200, which connects to the unresolved loop (residues 201–209). The average B -factors for NAD are 21 Å² for the adenine, 22 Å² for the adenine ribose, 21 Å² for the pyrophosphate, 32 Å² for the nicotinamide ribose, and 36 Å² for the nicotinamide.

Description of the NADH oxidase/flavine reductase fold

FRP exhibits the NADH oxidase/flavine reductase $\alpha + \beta$ fold (Murzin, 1996). Other enzymes with this fold include NADH oxidase from *Thermus thermophilus* (NOX, Protein Data Bank (PDB) entry 1nox; Hecht et al., 1995) and the major NAD(P)H:FMN oxidoreductase of *Vibrio fischeri* (FRase I, PDB entry 1vfr; Koike

et al., 1998). The differences among the FRP, NOX, and FRase I structures suggest that FRP belongs to one family, while NOX and FRase I belong to another family (Koike et al., 1998). This classification makes sense in terms of dinucleotide specificity: FRP prefers NADPH as substrate, whereas NOX and FRase I accept either NADPH or NADH. Sequence analysis suggests that the major NADPH-dependent nitroreductase of *Escherichia coli* (NfsA; Zenno et al., 1996a) belongs to the FRP family, and a minor nitroreductase of *E. coli* (NfsB; Zenno et al., 1996b) belongs to the NOX/FRase I family.

The FRP, NOX, and FRase I crystal structures are dimers of interlocking subunits with the active site located in the dimer interface (Fig. 1). FRP and NOX superimpose with an RMS difference of 1.4 Å for 294 equivalent α -carbon atoms (Fig. 2), while NOX and FRase I superimpose to 1.2 Å for 246 equivalent α -carbon atoms (Koike et al., 1998). The subunit structure of FRP has two main features. The first is an α - β - α sandwich consisting of a central four-stranded antiparallel β -sheet flanked by helices B and F on one side and by helices C, D, and G on the other side (Fig. 1). The second feature is an excursion domain that reaches out from one-half of the dimer and embraces the other half. The smaller part of the excursion domain is the N-terminal helix A, which contacts helices B and F of the other subunit (Fig. 1). The larger part of the excursion domain begins with a 14-residue stretch of mostly unstructured polypeptide chain that connects strand 4 of one subunit to a short β -strand that runs parallel to strand 1 of the other subunit. Given the intertwined nature of the dimer, FRP, NOX, and FRase I are probably examples of three-dimensional domain-swapped dimers (Bennett et al., 1995; Schlunegger et al., 1997).

There are three major secondary and tertiary structural differences between the FRP/NfsA and NOX/FRase I/NfsB families (Figs. 1, 2). First, the 10-residue loop that connects helices E and F of FRP is replaced by about 30 residues that form a loop-helix-loop motif in NOX and FRase I. Second, FRP contains a 3_{10} helix above the flavin ring system, which is absent in NOX and FRase I. Third, the NOX and FRase I polypeptide chains end after β -strand 5. Thus, NOX and FRase I lack FRP's helix I, the disordered 200s loop, and helices J and K. Note, however, that there is some overlap between FRP helix J and NOX's inserted helix (Fig. 2).

Fig. 1. Stereo view of the FRP dimer viewed down the molecular twofold axis with the B subunit yellow, A subunit red, FMN green, and NAD cyan. Figure prepared with MOLSCRIPT (Kraulis, 1991) and Raster3d (Merritt & Murphy, 1994).

Fig. 2. Stereo view of the α -carbon traces of FRP (heavy lines) and NOX (thin). The orientation is identical to that of Figure 1. The side chains of three FRP residues that are important for NAD binding are included: Asn134, Arg225, and Lys219.

Description of the FRP active site and the folded NAD

The active sites (two per dimer) are located in the dimer interface, with the isoalloxazine of the FMN cofactor wedged between β -strand 3 of one subunit and the 40s loop of the opposite subunit (Fig. 1). The entire *si* face of the isoalloxazine and both faces of the dimethyl benzene moiety are buried. Therefore, substrates are engaged at the *re* face of the pyrimidine portion of the isoalloxazine. Solvent accessibility calculations suggest that N1 and N5 of the isoalloxazine are candidates for receiving the hydride from NADPH C4 in the first step of catalysis (Tanner et al., 1996b).

The FMN cofactor is stabilized by several hydrogen bonds with the protein, and these are unchanged upon NAD binding (Fig. 3)

(Tanner et al., 1996b). The pyrimidine portion of the flavin hydrogen bonds to side chains of Arg15, Tyr69, Gln67, and to the main chain of the 130s 3_{10} helix. The FMN ribityl chain is hydrogen bonded to Arg15 of one subunit and to the main chain of Ser39 of the other subunit. Lys167 interacts with the ribityl chain, but the interaction is just outside our 3.5 Å distance cutoff. The FMN cofactor phosphate is anchored by hydrogen bonds to Arg169, His11, and Ser13.

NAD adopts a compact, folded conformation, with its pyrophosphate moiety next to the isoalloxazine and its nicotinamide and adenine moieties facing away from the flavin (Fig. 3). The distance between the nicotinamide C4 and FMN N1 atoms is 9.8 Å, which is much too long for hydride transfer. The bound NAD displaces an

Fig. 3. Stereo view of the FRP/NAD complex active site, with the B-subunit yellow, A-subunit red, FMN green, NAD cyan, and a protein molecule related by crystallographic symmetry blue. The dotted lines denote electrostatic interactions within 3.5 Å. Some FMN-protein interactions within 3.5 Å have been omitted for clarity.

inorganic phosphate ion that was found in the native FRP structure (Tanner et al., 1996b), with the nicotinamide phosphate occupying the same location as the displaced inorganic phosphate. We note that a water molecule, rather than a phosphate ion, is bound at this location in NOX and FRase I.

NAD is folded such that the nicotinamide ring and the adenine five-membered ring stack in a nearly parallel fashion. The inter-ring distance is 3.6 Å, based on ring centroids. This arrangement suggests that π - π interactions (Hunter et al., 1991) help to stabilize the observed conformation of NAD. The conformation of the NAD in our structure is the most compact observed to date, based on calculations of the radius of gyration and the distance between the adenine C6 and nicotinamide C2 atoms (Table 1; Fig. 4). The latter distance is commonly used as a measure of NAD(P) compactness (Bell et al., 1997).

Several electrostatic interactions stabilize the NAD (Fig. 3). The pyrophosphate hydrogen bonds to the main chain and side chain of Ser 41, the ribityl chain of the cofactor FMN, a water molecule (402) with B -factor 29 Å², and Arg225. Water 402 and Arg225 participate in a salt link-hydrogen bond network involving Lys219, water 369 (B = 19 Å²), Glu99, and Arg133. Glu99 and Arg133 form an intersubunit salt link. The adenine ribose ring of NAD is stabilized by hydrogen bonds to Asn134 and Gly65. Finally, the pyrophosphate might interact with the π system of the isoalloxazine, based on the report of a similar phosphate:aromatic interaction in the crystal structure of a complex of triethyl phosphate and benzotrifurazan (Cameron & Prout, 1972).

Only the B-chain active site contains NAD due to blockage of the A-chain active site by the B-chain 180s loop of a symmetry-related dimer. Specifically, χ_1 of Asn A134 differs from that of Asn B134, which allows Asn A134 to hydrogen bond to a symmetry mate of Gln B180, rather than hydrogen bonding to Arg A225. Arg A225, in turn, salt links to a symmetry mate of Asp B187. Note that in the B-subunit, which does bind NAD, Arg B225, and Asn B134 hydrogen bond directly to the NAD and to each other (Fig. 3).

NAD also interacts with the A-subunit 180s loop of a symmetry-related protein molecule (twofold screw along y followed by cell translations along a and c). The adenine interacts with the main chain of Leu184 and Leu186 (Fig. 3). Its ribose hydrogen bonds with Asp187. The nicotinamide hydrogen bonds to the main chain of Leu184 and the side chain of Glu183. The nicotinamide ribose hydrogen bonds with Glu183.

Discussion

Novelty and importance of the observed NAD conformation

The FRP/NAD structure reveals a new conformation of an important and ubiquitous biochemical substrate and coenzyme. NAD adopts a folded conformation with parallel ring stacking in our structure. In contrast, NAD(P) bound to enzymes typically adopts an extended conformation (Fig. 4, see Bell et al., 1997, for review). Perhaps the most recognizable example is NAD bound to a Rossmann fold domain (Brändén & Tooze, 1991). The NAD in diphtheria toxin and the NADP of catalase are more compact than those bound to a Rossmann fold (Table 1; Fig. 4); however, they are more extended than the NAD in our structure, and neither molecule exhibits ring stacking.

The disposition of the nicotinamide with respect to the isoalloxazine is another unique feature of the FRP/NAD structure. The

Table 1. Measures of compactness for six NAD(P) molecules

Structure (data bank ID)	Radius of gyration (Å)	C6A-C2N distance (Å)
FRP (2bkj)	4.2	3.9
Catalase (8cat)	4.6	8.3
Li ⁺ complex (nadlih10)	5.5	12.4
Diphtheria toxin (1tox)	5.5	12.4
GAP dehydrogenase (1cer)	6.1	14.7
Glutathione reductase (1geu)	6.7	17.9

Fig. 4. Conformations of NAD(P) bound to (from top to bottom) flavin reductase P, catalase (Fita & Rossmann, 1985), diphtheria toxin (Bell et al., 1997), and glyceraldehyde-3-phosphate dehydrogenase (Tanner et al., 1996a). Note that catalase binds NADP.

pyrophosphate moiety is adjacent to the flavin ring system and the nicotinamide C4 atom is 10 Å from the FMN N1 atom. This arrangement is inconsistent with hydride transfer and has not been observed in enzyme structures. Although there are no other structures in the Protein Data Bank containing an FMN interacting with NAD(P), there are several structures containing FAD and NAD(P), including glutathione reductase (Mittl et al., 1993, 1994), lipamide dehydrogenase (Mattevi et al., 1992), NADH peroxidase (Stehle et al., 1993), thioredoxin reductase (Waksman et al., 1994), trypanthione reductase (Bailey et al., 1993), and ferredoxin–NADP reductase (Serre et al., 1996). In these structures, NAD(P) binds in an extended conformation with its nicotinamide 3.5 Å above, and nearly parallel to, the isoalloxazine (Mittl et al., 1993, 1994).

A crystal structure of NAD in the absence of protein has also been determined. NAD adopts an extended conformation in the crystal structure of the Li^+ /NAD complex (Saenger et al., 1977). The degree of compactness is similar to that of the NAD in diphtheria toxin (Table 1). Ring stacking, similar to that observed in our structure, is also seen in the Li^+ /NAD complex; however, the stacking is intermolecular rather than intramolecular.

The solution structure of NAD has been examined; however, a high resolution model is not available. Spectroscopic studies suggest that NAD exists in solution as an equilibrium mixture between a folded conformation with parallel ring stacking and an extended nonstacked form (Jardetzky & Wade-Jardetzky, 1966; Miles & Urry, 1968; Oppenheimer et al., 1971). Molecular mechanics calculations also suggest that ring stacking is the hallmark of the folded conformation of free NAD (Thornton & Bayley, 1977). Because our structure displays ring stacking, it might resemble the folded conformation that NAD adopts in solution. Furthermore, it might be possible to use our structure as input to computational studies of NAD in solution and enzyme–NAD encounters.

The FRP/NAD structure provides new information about the conformational diversity of NAD, and together with the extended NAD structures, provides insight into the role of NAD flexibility in catalysis. These data suggest that enzymes are tuned to stabilize extended conformations of NAD and that only extended NAD molecules are ready for catalysis. Extended conformations have two main advantages over a folded conformation. First, an extended substrate exposes more surface area; thus, there are more sites available to interact with the enzyme. Second, by unfolding, NAD reduces the steric bulk around the nicotinamide, thus allowing the nicotinamide to engage its hydride transfer partner with the correct orientation and at the required distance of less than 4 Å.

Possible implications for FRP catalysis

NAD appears to inhibit FRP by adopting a folded conformation. The active site crevice is too small to accommodate the stacked nicotinamide/adenine moiety; therefore, NAD enters the active site “backward,” with the pyrophosphate next to the flavin cofactor. This manner of binding is consistent with the observation of an inorganic phosphate in the native FRP structure, yet it is inconsistent with hydride transfer between the nicotinamide C4 and the isoalloxazine.

The binding of NAD to FRP differs significantly from the binding of the inhibitor dicoumarol to FRase I (Koike et al., 1998). One of the dicoumarol rings inserts in a parallel orientation between the FMN isoalloxazine and the side chain of Phe124. (Note that FRP does not have a residue analogous to Phe124 due to tertiary structural differences between FRP and FRase I.) The dicoumarol ring

is 3.4 Å from the isoalloxazine. The dicoumarol/FMN arrangement is similar to the NADP/FAD arrangement in glutathione reductase (Mittl et al., 1993, 1994), which suggests that dicoumarol inhibits FRase I by mimicking the catalytically competent conformation of the dinucleotide substrate.

The FRP/NAD structure could provide insight into the basis of FRP's dinucleotide substrate specificity. NADH would be an unsuitable substrate if it binds to FRP in a folded conformation. The reduced nicotinamide ring of a folded NADH would be unable to approach the isoalloxazine close enough (3.5 Å) for hydride transfer to occur due to steric clash between the protein and the adenine. This idea suggests that the basis for NADPH specificity involves recognition of the adenosine 2'-phosphate by the enzyme during the enzyme–substrate encounter and subsequent unfolding of the dinucleotide into its active conformation. Once NADPH is converted to NADP, the enzyme repels the now positively charged nicotinamide ring. In the reverse of substrate binding, interaction between the enzyme and the adenosine 2'-phosphate encourages the product to assume the folded conformation to facilitate its release.

The limited accessibility of the active site accounts for two important features of FRP catalysis. As discussed above, the active site is not large enough to allow the nicotinamide of a folded dinucleotide to approach the isoalloxazine for hydride transfer; therefore, the dinucleotide must be unfolded prior to catalysis. Second, the volume of the active site relates to the observation of ping-pong bisubstrate–biprodukt reaction kinetics (Tanner et al., 1996b). The active site is not large enough to simultaneously accommodate the NADPH nicotinamide and substrate flavin isoalloxazine. Thus, the sequence of catalytic events is (1) binding of an extended NADPH and reduction of the cofactor FMN, (2) release of NADP, and (3) binding of the oxidized flavin substrate. Therefore, modification of the active site volume could be used to alter the inhibition characteristics, substrate specificity, and kinetics of FRP.

It should be noted that the possibility cannot be eliminated that the folded conformation of the FRP-bound NAD has little or no direct bearing on the nature of NADPH binding by FRP. However, our preliminary data show that NAD at 5–20 mM significantly inhibited the activity of FRP in a Tris buffer. Such a finding further suggests that NAD and NADPH may share some common features in binding to FRP.

The FRP/NAD crystal structure also reveals structural differences between the FRP/NfsA and NOX/FRase I/NfsB families that might relate to dinucleotide substrate specificity. NOX and FRase I lack several of the interactions that stabilize the folded NAD in our structure, suggesting that NOX and FRase would not bind the folded form of NAD. Specifically, NOX (FRase I) has a Trp (Ile) in place of Ser41. NOX and FRase I lack Arg225 and Lys219 because of their shorter polypeptide chain length. NOX and FRase I do not have residues analogous to Arg133 and Asn134 because these residues reside on a 3_{10} helix in FRP that is not present in the NOX and FRase I. Last, NOX and FRase I do not have an analogue of Glu99 because of the secondary and tertiary structural differences in the region of the 90s loop.

How these structural differences modulate substrate specificity and reaction kinetics is unknown; however, the current structure and its comparison to other NADH oxidase/flavine reductase enzymes suggest several amino acids that could be mutated to help address these issues. Such work is ongoing. For example, mutation of Glu99 to Gly transforms NfsA from a nitroreductase to a flavin

reductase; however, the structural basis of this change is unclear because the mutant structure is not known (Zenno et al., 1998). Future biochemical and crystallographic studies of other mutants, with and without substrates and inhibitors bound, are needed to understand the structure–function relationships of the NADH oxidase/flavine reductase family.

Influence of crystal packing

It is difficult to assess the role of crystal packing forces in determining the observed NAD conformation. Nonetheless, two observations suggest that this folded NAD conformation primarily results from the affinity of the active site for pyrophosphate.

First, an inorganic phosphate was found in the active site of the native FRP structure at the same location as the nicotinamide phosphate in this structure. Phosphate binding to the active site does not occur in NOX or FRase I, suggesting that this aspect of FRP might be related to its specificity for NADPH. Second, the pyrophosphate appears to be the most tightly bound section of NAD, based on electron density maps and *B*-factors. Electron density indicating the position of the pyrophosphate was unambiguous in the first map calculated before NAD was included in the model (Fig. 5A). The density for nicotinamide and its ribose emerged later in the refinement and never reached the quality of that observed for the adenine half of the NAD (Fig. 5B). Correspondingly,

the *B*-factors are lowest in the pyrophosphate and highest in the nicotinamide, indicating that the pyrophosphate is the most rigidly bound part of the NAD.

Materials and methods

Crystallization and X-ray data collection

Purification, crystallization, and space group determination of FRP from *V. harveyi* cloned in *E. coli* were described elsewhere (Tanner et al., 1994). The native enzyme, which includes two FMN and two inorganic phosphate ions per dimer, was crystallized at room temperature in sitting drops equilibrated over a reservoir solution containing 30% poly(ethylene glycol) 6000 and 0.1 M HEPES pH 7.0. The space group is $P2_1$ with cell dimensions $a = 51.2$ Å, $b = 85.9$ Å, $c = 58.1$ Å, $\beta = 109.3^\circ$. The asymmetric unit contains one homodimer.

To prepare crystals of the NAD-complexed enzyme, native crystals were soaked in 30% poly(ethylene glycol) 6000, 0.1 M HEPES, 35 mM NAD, pH 7.0 for 48 h. The unit cell parameters were initially determined as $a = 51.2$ Å, $b = 85.9$ Å, $c = 60.0$ Å, $\beta = 116.2^\circ$. However, reassignment of the *c*-axis along $(-1\ 0\ -1)$ and the *b*-axis along $(0\ -1\ 0)$ with respect to the initial unit cell yields $a = 51.2$ Å, $b = 85.9$ Å, $c = 59.2$ Å, $\beta = 114.7^\circ$. Both cell choices are similar to that of the native enzyme structure, and the latter was selected owing to its close isomorphism with the native structure.

Fig. 5. Stereo views of $2F_o - F_c$ electron density maps (contoured at 1σ) draped over the final, refined model of NAD. **A:** Map calculated before NAD was included in the model, with $R_{\text{test}} = 24.9\%$, $R_{\text{work}} = 23.9\%$ for 6.0–2.08 Å data. **B:** Map calculated from the final refined structure, with $R_{\text{test}} = 20.6\%$ and $R_{\text{work}} = 18.5\%$ for 32.0–2.08 Å data. Figure prepared with BOBSCRIPT (Esnouf, 1997) and Raster3d (Merritt & Murphy, 1994).

Data were collected to 2.08 Å resolution at 14 °C using a FAST area detector coupled to a Rigaku rotating anode operating at 50 kV and 180 mA with a 0.5 mm collimator. Three scans, totaling 360°, were collected with the detector distance at 80 mm and frame width of 0.1°. These data sets were processed with MADNES (Messerschmidt & Pflugrath, 1987) and XSCALE (Kabsch, 1988). The merged data set is 84% complete for 32–2.08 Å data with an R_{sym} on I of 3.4%. The 2.3–2.2 Å shell of data is 84% complete, while the 2.2–2.08 Å shell is 38% complete.

Molecular replacement and structure refinement

Rigid body refinement starting from the native structure failed because of the unit cell changes induced by the binding of NAD. Therefore, phases were determined by molecular replacement using AMoRe (Navaza, 1994) with the 1.8 Å native structure serving as the search model. FMN, inorganic phosphate, and water molecules were removed prior to molecular replacement. AMoRe calculations at 15–3.5 Å gave a solution with an R -factor of 28% and a correlation coefficient of 76%. After accounting for an arbitrary translation along the y axis in $P2_1$, the transformation from AMoRe that moves the uncomplexed structure onto the NAD-complexed structure is a rotation of 3.3° about an axis with direction cosines (0.998, 0.012, 0.066) followed by a translation in orthogonal space of $x = -0.11$, $y = -0.83$, $z = 0.97$ Å. The initial map calculated from molecular replacement phases showed electron density for the NAD in the B-chain active site, while crystal contacts prevent NAD from binding in the A-chain active site.

Conjugate gradient positional refinement was performed using X-PLOR v3.851 (Brünger, 1992) and the Engh and Huber (1991) force field. Hydrogen atoms were deleted and electrostatic forces were disabled. Cross-validation was used with a test set of reflections chosen to match as close as possible the test set used during refinement of the native structure. The test set contained 2,159 reflections, while the working set contained 21,439 reflections. The X-ray weight W_a was determined by minimization of R_{test} and set to 100,000. O (Jones et al., 1991) was used for model building.

Refinement and model building were carefully monitored using R_{test} , and B -factor refinement was reserved for the latter stages of refinement. Following molecular replacement, FMN was added to both active sites along with 51 water molecules taken from the 107 water molecules of the native structure. The R -factors after positional refinement were $R_{\text{test}} = 24.9\%$ and $R_{\text{work}} = 23.9\%$ for 6.0–2.08 Å data. Difference maps calculated at this stage indicated the location of the pyrophosphate and adenine moieties of NAD (Fig. 5A). The pyrophosphate of NAD was added to active site B, which lowered the R -factors to $R_{\text{test}} = 24.5\%$ and $R_{\text{work}} = 23.0\%$. The adenine and its ribose were added, resulting in $R_{\text{test}} = 24.3\%$ and $R_{\text{work}} = 22.5\%$. The side chain of Glu A183 and 20 water molecules were added, which lowered the R -factors to $R_{\text{test}} = 23.8\%$ and $R_{\text{work}} = 21.8\%$. A bulk solvent mask was calculated using 32–2.08 Å data, followed by positional refinement, overall B -factor refinement, and individual B -factor refinement. Note that up to this point the B -factors of the native structure had been used. The resulting R -factors were $R_{\text{test}} = 21.0\%$ and $R_{\text{work}} = 18.8\%$. Next, the nicotinamide and its ribose were added, which lowered the R -factors to $R_{\text{test}} = 20.9\%$ and $R_{\text{work}} = 18.6\%$. Finally, the side chains of Lys B16 and Lys B167 were repositioned, and the side chains of Lys A219, Lys B219, Asp B92, and Glu B138 were added to the model along with two more water molecules.

The native FRP structure with inorganic phosphate bound is available from the PDB as entry 1bkj. The coordinates and structure factors of the FRP/NAD complex have been deposited as entries 2bkj and r2bkjsf, respectively.

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