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A variety of electrostatic interactions and adducts can activate NAD(P) cofactors for hydride transfer

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ABSTRACT

In NAD(P)-dependent enzymes the coenzyme gives or takes a hydride ion, but how the nicotinamide ring is activated to form the transition state for hydride transfer is not clear. On the basis of ultra-high resolution X-ray crystal structures of liver alcohol dehydrogenase (LADH) in complex with NADH and a number of substrate analogues we proposed that the activation of NADH is an integral part of the enzyme mechanism of aldehyde reduction [R. Meijers, R.J. Morris, H.W. Adolph, A. Merli, V.S. Lamzin, E.S. Cedergren-Zeppezauer, On the enzymatic activation of NADH, The Journal of Biological Chemistry 276(12) (2001) 9316–9321, %U http://www.ncbi.nlm.nih.gov/pubmed/11134046; R. Meijers, H.-W. Adolph, Z. Dauter, K.S. Wilson, V.S. Lamzin, E.S. Cedergren-Zeppezauer, Structural evidence for a ligand coordination switch in liver alcohol dehydrogenase, Biochemistry 46(18) (2007) 5446–5454, %U http://www.ncbi.nlm.nih.gov/pubmed/17429946]. We observed a nicotinamide with a severely distorted pyridine ring and a water molecule in close proximity to the ring. Quantum chemical calculations indicated that (de)protonation of the water molecule can be directly coupled to activation of NADH for hydride transfer. A systematic search of the Protein Data Bank (PDB) for atoms that come within van der Waals distance of the pyridine ring of the nicotinamide reveals that a large number of NAD(P)-containing protein complexes are involved in electrostatic interactions with the enzymatic environment. Using the deposited diffraction data to analyze the cofactor and its surroundings, we observe several adducts between protein atoms and the pyridine ring that were not previously reported. This further indicates that the enzymatic activation of NAD(P) induced by electrostatic interactions is an essential part of the hydride transfer mechanism.

1. Introduction

Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) play a crucial role in the metabolism of the cell. The NAD/NADH redox couple is stable in solution and the ratio is used to inform many cellular processes on the metabolic state of the cell [3,4]. The ratio is modified by enzymes that use NAD(P) as a cofactor to transfer energy. How do enzymes activate NAD(P) so that it engages in hydride transfer with the substrate? The oxidized cofactor NAD+ has been crystallized and the structure revealed that the pyridine ring of the nicotinamide moiety which contains the C4 carbon that acts as hydride acceptor is flat and therefore aromatic [5]. To date, no structure for the reduced NADH cofactor alone has been reported, but based on theoretical studies, it is expected that the pyridine ring assumes a boat conformation [6]. High-resolution crystal structures of enzyme bound NADH confirm this conformation [7–9]. It is assumed that the reduced ring is no longer aromatic and the ring density is localized to accommodate the hydride at C4. Theoretical calculations suggested that the distortion of the pyridine ring into a boat conformation provides sufficient energy to induce hydride transfer [6]. These calculations where done on a free NADH molecule *in vacuo* and we confirmed with quantum chemical calculations with an improved $6-31+G^{**}$ basis set that the boat conformation exists [1]. Theoretical studies on the role of enzyme motion on the reaction coordinate for hydride transfer also identify the puckering of the pyridine ring into the boat conformation as favorable for hydride transfer [10]. However, if the boat conformation can exist *in vacuo* and probably as well in solvent, spontaneous hydride transfer would occur without any specific catalytic intervention, which is not likely. The deformation of the pyridine ring in a boat form is therefore in itself not sufficient to trigger hydride transfer.

In many enzyme systems, it has been observed that the UV–vis spectrum of the reduced cofactor changes when the cofactor is enzyme bound [3,11]. Since these spectral changes can be directly related to a perturbation of the pyridine ring of the NAD(P)H

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cofactor, it is apparent that the enzymatic environment does affect the pyridine ring. Circular dichroism (CD) and circular polarization of luminescence (CPL) spectra also show a shift when free NADH and enzyme bound NADH are compared [12,13]. It was thought that in the 'free' NADH molecule, the adenosine ring stacks onto the nicotinamide, and the extension of the NADH molecule upon enzyme binding might explain the shift. However, hydrolysis of the pyrophosphate bond in NADH has little effect on the fluorescent decay times, discarding the extension of the NADH molecule as a source of the spectral shift [12]. It was also found in liver alcohol dehydrogenase that there is a difference between the CD and CPL spectra of the binary NADH–LADH complex and the ternary complex with the inhibitor isobutyramide (IBA) [13]. The spectrum of the binary complex indicates a continuous dynamic restructuring close to the pyridine ring, whereas the IBA ternary complex is more rigid. Ultra-high resolution structures of a binary NADH–LADH complex revealed that indeed a dynamic structure can be observed, where the metal bound water molecule switches between two positions [1]. One position corresponds to the substrate-binding site. The second position situates the water molecule in close proximity to the pyridine ring. The pyridine ring is heavily distorted and the electron density inside the ring is delocalized. The ultra-high resolution structure of the ternary complex with IBA confirms a more rigid active site [2]. The water molecule has been replaced by the inhibitor, and the pyridine ring conformation has relaxed into a boat. This conformation is similar to what was previously shown for ternary inhibition complexes [7–9]. Interestingly, the electron density inside the pyridine ring is now aromatic, which illustrates the inhibitive effect of IBA on the NADH cofactor transition state.

These observations have raised the question whether the presence of a dynamic water molecule that forms an adduct with the pyridine ring is an anomaly, or whether this phenomenon is found in other NAD(P)-dependent enzymes. To address this question, we have performed a systematic evaluation of the enzymatic environment of the pyridine ring of the nicotinamide moiety among NAD(P) containing protein structures deposited in the Protein Data Base (PDB) [14]. The survey resulted in a number of interesting configurations where the enzyme clearly influences the transition state of the cofactor through close electrostatic interactions between the pyridine ring and the surrounding protein atoms.

2. Methods

The PDB was scanned to extract all entries containing either NAD or NADP. To ensure that the NAD(P) environment was well-resolved, we further selected only those entries for which X-ray diffraction was reported to extend to a resolution better than 2.0 Å. A *Python* program was written to find any atoms situated within van der Waals radius of the six atoms that constitute the pyridine ring. Within van der Waals radius is defined here as C–O < 3.2 Å, C–N < 3.3 Å, C–C < 3.4 Å and C–S < 3.5 Å. Redundant close contacts were removed (if the same interaction was observed in more than one protein molecule in the asymmetric unit), and only the shortest distance between the pyridine ring and the atom in proximity is reported. Structures of interest were further investigated if deposited structure factors were available using the Electron Density Server [15]. Pymol was used to make the figures [16]. The PDB entry 1UWL was refined with *Phenix* [17] using the deposited diffraction data with a modified restraint library for the NAD cofactor without planar restraints on the pyridine ring and relaxed penalties on the aromatic bond and angle restraints of the ring. The *R* factor obtained with the modified refinement protocol is 16.0% (*R*-free 18.8%), which is close to the reported *R* factor 16.8% (*R*-free 18.9%).

3. Results

3.1. A systematic survey of the PDB

A total of 340 entries were found to contain a NAD(P) cofactor and to be based on diffraction data better than 2.0 Å. A total of 15 sulfur, 18 nitrogen, 47 carbon and 153 oxygen atoms were found to reside within van der Waals distance from the pyridine ring (Table 1). It is important to note the discrepancy between the total number of clashing atoms, and the number of PDB files that show clashes. Most PDB entries that are affected have more than one clash per nicotinamide moiety. In the case of the most prominent clashing type, between an oxygen atom and the pyridine ring, there are on average two clashes per nicotinamide. This can in large part be explained by the protocol that is followed during the refinement of the structure against the X-ray data. Most structures would lose their chemical consistency if they were refined only against the X-ray data. A library of restraints is used to keep the atoms at a chemically sensible position. The library contains standard bumping restraints that are used to keep atoms apart that are not considered to interact with each other. These bumping restraints use quite conservative van der Waals radii. For instance, during a standard refinement in Refmac5, oxygen atoms are kept at a minimal distance of 3.6 Å from the pyridine ring [18]. If the electron density steers the refinement of the atom position to get closer to the ring, the bumping restraint forces it back to the standard minimal distance. In the 75 PDB entries that do allow oxygen atoms to come within van der Waals distance of the ring (here defined as closer than 3.2 Å), the refinement protocol must have allowed lower penalties on the bumping restraints, and the weight given to the X-ray data is higher. It is likely that among the PDB entries that do not show any clashes, some entries have been affected by the bumping restraints keeping atoms at their minimal theoretical distance that should in fact lie close to the pyridine ring.

Given the refinement restraints applied to each NAD(P) protein complex, it is remarkable to find a large number of atoms so close to the pyridine ring that they will have a strong effect on the ring conformation. Below is a short description of the outliers that come within 3.0 Å from the pyridine ring of the nicotinamide.

Nitrogen outliers: In PDB entry 1M2W, the ND1 atom of Asn 191 is situated at 2.9 Å from the NC2 atom of the pyridine ring. In PDB entry 2C54, a disordered Lys 217 overlaps with the disordered nicotinamide moiety.

Carbon outlier: In PDB entry 2CFC, Pro 185 stacks sideways above the NC5 atom of the ring, and the CB atom lies 3.0 Å from NC5. In the same structure, a water molecule is situated at 2.9 Å from the NC4 atom of the pyridine ring, a carbonyl oxygen from Gly 186 is

Table 1
PDB survey of protein atoms within van der Waals distance of the pyridine ring of NAD(P).

Interaction	C–O	C–S	C–N	C–C
Total PDB entries	75	14	18	34
Number of close contacts	153	15	18	47
3.5–3.4 Å	–	5	–	–
3.4–3.3 Å	–	4	–	33
3.3–3.2 Å	–	1	11	8
3.2–3.1 Å	65	1	4	5
3.1–3.0 Å	48	2	1	0
3.0–2.9 Å	17	0	2	1
2.9–2.8 Å	10	0	0	0
2.8–2.7 Å	7	0	0	0
2.7–2.6 Å	0	0	0	0
<2.6 Å	8*	2	0	0

* Five contacts are related to the hydroxide adduct with NADH in liver alcohol dehydrogenase [1,2].