marker is known to be near the centromere (*19*), the association of PRP loci and the *Gr-1* locus on a chromosome fragment in conjunction with the congenic mice experiment described above, suggests that the PRP loci may be in the proximal (toward the centromere), and not in the central, region of chromosome 8.

Some mouse chromosomal 8 genes, such as, *Gr-1*, *Got-2*, *Prt-2*, and *Aprt* have counterparts on human chromosomes 8 and 16 (*20*). Human PRP genes have now been localized to chromosome 12 rather than 8 or 16 (*4*). The reasons for maintenance or disruption of linkage groups during evolution are unknown, although closely linked genes tend to be conserved (*20–22*).

In previous studies, the structural or regulatory genes for salivary proteins in the mouse have not been found on chromosome 8, but occur on at least four different chromosomes (*23–26*) including chromosome 1 (renin), chromosome 3 (amylase and nerve growth factor), chromosome 2 (parotid secretory protein), and chromosome 7 (tamase and other salivary proteins).

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## Conformational Variability of $NAD^+$ in the Free and Bound States: A Nicotinamide Sandwich in $NAD^+$ Crystals

Abstract. *X-ray analysis of the free-acid crystal form of the coenzyme nicotinamide adenine dinucleotide ($NAD^+$) revealed a conformational difference between the free $NAD^+$ molecule and one bound in enzymes or complexed to $Li^+$ ions. The pyrophosphate group showed asymmetry in the phosphate-oxygen bonds of the phosphate-oxygen-phosphate link; this bond at the nicotinamide side of the link is longer than that at the adenosine side by 0.04 angstrom. The crystal structure showed a novel intermolecular stacking of adenine and water molecules on opposite sides of nicotinamide that gives rise to a nicotinamide sandwich.*

The coenzyme nicotinamide adenine dinucleotide ($NAD^+$), formerly called $DPN^+$, coenzyme I, cohydrase I, or cozymase, plays a dominant role in the transfer of hydride (a proton and two electrons) in biological reduction-oxidation (redox) processes. Along with the other coenzyme flavin adenine dinucleotide, $NAD^+$ is responsible for most enzyme-catalyzed dehydrogenation reactions. It consists of adenylic acid and nicotinamide-5′-ribonucleotide linked through a 5′-5′ pyrophosphate linkage (Fig. 1); the nicotinamide ring system is redox active, while the adenylic acid seems to serve mainly as a handle for binding the coenzyme. In about 250 known enzymatic redox reactions involving $NAD^+$ the reactive site is at C-4, and 1,4-dihydronicotinamide is the exclusive isomer formed biologically. Ever since the elucidation of its chemical structure in 1936, numerous studies have been carried out to determine the three-dimensional conformation of $NAD^+$ in the free and bound states (*1–4*). It was inferred to have a folded conformation in aqueous solutions (*5*), and x-ray crystallographic studies of $NAD^+$ bound to several dehydrogenases at low resolution showed an extended form, with the nucleotides exhibiting nonstandard, higher energy conformations (Fig. 1 and Table 1) (*4*). X-ray analysis of $Li^+$-$NAD^+$ complex (*3*, *6*) at medium resolution showed an extended conformation for the $NAD^+$ molecule. We now describe our high-resolution x-ray study of the free-acid form of $NAD^+$, in which the $NAD^+$ molecule adopts a conformation strikingly different from that found for $NAD^+$ when it is bound to enzymes or complexed to $Li^+$ ions.

The crystal structure of $NAD^+$ shows interesting chemical, conformational, and stacking features. In this structure N-1 of adenine is protonated, so both the purine and pyrimidine bases are positively charged; both phosphates are ionized, and each one carries a negative charge. If the differences in the heterocycles are neglected, $NAD^+$ has a pseudo diad axis passing through the pyrophosphate oxygen, OPP. However, the two phosphates are not chemically equivalent. The PN–OPP bond of 1.617(3) Å is significantly longer than the PA–OPP bond of 1.578(2) Å. The P–O–P angle is 133.3(2)°, a value similar to that found in other pyrophosphates (*3*). The longer P–O bonds, together with a wider P–O–P angle, allow considerable flexibility in the pyrophosphate links. Another interesting chemical feature in this structure is the pronounced bond-shortening anomeric effect in the C–O bonds of the ribose rings (*7*, *8*); the C-1′–O-4′ and C-4′–O-4′ bond lengths are 1.380(5) and 1.458(5) Å, respectively, for the ribose moiety attached to nicotinamide and 1.404(5) and

Table 1. Comparison of the conformation of $NAD^+$ and its analogs in the free and enzyme-bound states. Data were obtained from x-ray crystallographic or nuclear magnetic resonance studies of lactate dehydrogenase (LDH) (*22*), malate dehydrogenase (MDH) (*23*), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from lobster (*24*) or *Bacillus stearothermophilus* (*25*), (*S*)-lac-NAD-LDH complex (*26*), liver alcohol dehydrogenase–adenosine diphosphoribose complex (LADH) (*19*), and sorbitol dehydrogenase (SDH; data from nuclear Overhauser effect studies) (*27*). A, *anti*; S, *syn*; g, gauche; t, *trans*.

| Analog or complex | Adenosine moiety | | | Nicotinamide moiety | | | Overall | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\chi_{CN}$* | Sugar pucker† | C-4′–C-5′ bond | $\chi_{CN}$* | Sugar pucker‡ | C-4′–C-5′ bond | Conformation | A and N rings | |
| | | | | | | | | Distance (Å) | Angle (degrees) |
| $NAD^+$ (free acid) | A | C-2′ | $g^+$ | A | C-2′ | $g^+$ | Folded | 9.6 | 14 |
| $NAD^+$-$Li^+$ | A | C-2′ | $g^+$ | A | C-3′ | $g^+$ | Extended | 11.5 | 113 |
| $NAD^+$-$LDH^+$ | A | C-3′ | $g^-$ | A | C-3′ | $g^-$ | Extended | 14 | 58 |
| (*S*)-lac-$NAD^+$-$LDH^+$ | A | C-3′ | t | A | C-2′ | $g^+$ | Extended | 14.8 | 47 |
| MDH | A | C-3′ | $g^-$ | A | C-2′ | $g^-$ | Extended | 14.3 | 80 |
| Lobster GAPDH | | | | | | | | | |
| Red subunit | A | C-2′ | $g^-$ | S§ | C-3′ | $g^+$ | Extended | 15 | 73 |
| Green subunit | S | C-3′ | t | S§ | C-2′ | $g^+$ | Extended | 15 | 82 |
| *B. stearothermophilus* GAPDH | A | C-2′ | $g^-$ | S§ | C-2′ | $g^+$ | Extended | | |
| LADH | A | C-3′ | t | | C-3′ | t | Extended | | |
| SDH | A | C-3′ | $g^+$ | A | C-1′ | t | Folded | 8.5 | 120 |

*Conformation measured across the glycosidic bond. †All sugar puckers were *endo* in the adenosine moiety. ‡All sugar puckers were *endo* in the nicotinamide moiety except for SDH, which was *exo*. §GAPDH is a B-type dehydrogenase.

1.443(5) Å, respectively, for the ribose of adenosine.

The $NAD^+$ molecule takes one of the lowest possible energy conformations for both the 5′ nucleotides and for the pyrophosphate link (Fig. 1). The conformation of both the 5′ nucleotides is standard (*9*) and may be summarized as follows: *anti* across the glycosidic bonds [O-4′N–C-1′N–N-1N–C-6N, 30.3(6)°; O-4′A–C-1′A–N-9A–C-8A, 34.3(5)°], gauche plus ($g^+$) across C-4′–C-5′ bonds [48.2(5)° for nicotinamide riboside and 54.0(5)° for adenosine], an *endo* sugar pucker at C-2′ for both riboses, and the phosphate group *trans* to C-4′ in both nucleotides [see (*9*)]. Looking along the virtual P . . . P bond, the phosphate groups are staggered in this structure, but in the $Li^+$-$NAD^+$ complex they are eclipsed because of the $Li^+$ coordination of O-1A and O-1N. The C–$NH_2$ bond of the carboxamide group is *trans*-planar with the C-3N–C-4N bond in this structure as well as in the unsubstituted nicotinamide (*10*); in most other N-1–substituted nicotinamides, including the $Li^+$-$NAD^+$ complex, the C–$NH_2$ bond is *cis*-planar (*6*).

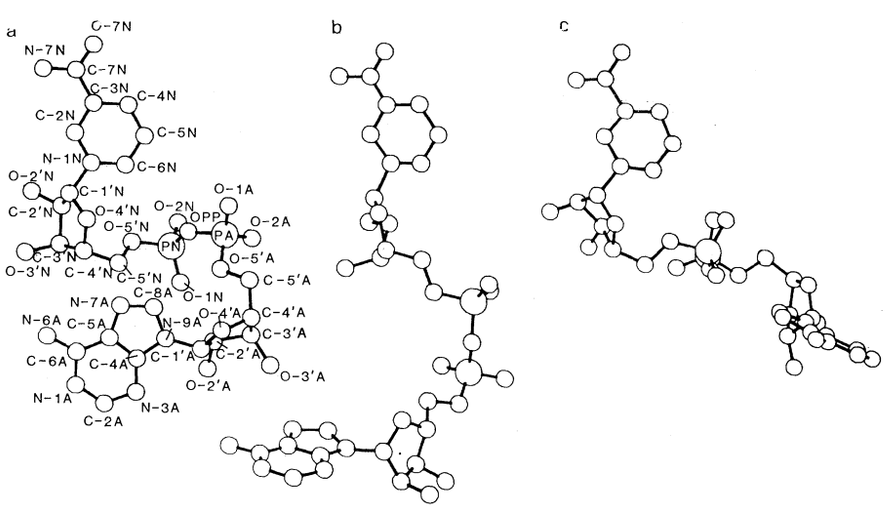


Fig. 1. Conformation and numbering of $NAD^+$ in the free-acid form (a), complexed to malate dehydrogenase (b), and complexed to $Li^+$ (c). $NAD^+$ consists of two nucleotides, adenylic acid and nicotinamide-5′-ribonucleotide, linked through a 5′-5′ pyrophosphate link. Crystals of $NAD^+$ were obtained at *p*H 2.9 from aqueous solutions equilibrated with acetone. The same crystal form was obtained even when the *p*H was raised to 4.0 with 1*N* LiOH, NaOH, or KOH [a condition at which the $Li^+$-$NAD^+$ complex was obtained by other investigators (*3*)]. A crystal (0.25 by 0.10 by 0.30 mm) was mounted with mother liquor in a capillary tube and used for all x-ray studies on an ENRAF-NONIUS CAD-4 diffractometer. Crystals of $NAD^+$-tetrahydrate ($C_{21}H_{27}N_7O_{14}P_2 \cdot 4H_2O$) are triclinic [*a*, 8.643 (2) Å; *b*, 8.857(1) Å; *c*, 11.184(3) Å; α, 109.74(2)°; β, 90.76(2)°; γ, 103.43(1)°; volume, 779.9 $Å^3$; $Z = 1$; space group $P\bar{1}$]. Full three-dimensional data of 3460 reflections (2624 ≥ 3 standard deviations) to the limit of the Cu sphere for Cu-$K_\alpha$ were collected by the $\omega - 2\theta$ scan method. The structure was solved by means of Patterson and difference-Fourier techniques and refined by a full-matrix least-squares procedure to an *r* value of 0.03. All the 45 hydrogen atoms were located in difference electron density maps, and their positional and thermal parameters were included in the refinement. The average of estimated standard deviations in P–O, C–O, C–N, C–C, and R–H (where R is any nonhydrogen atom) bond distances are 0.0025, 0.005, 0.005, 0.006, and 0.06 Å, respectively; in P–O–P, R–R–R, and R–R–H the bond angles are 0.16, 0.46, and 5°, respectively.

The crystal structure of $NAD^+$ does not show any intramolecular stacking of adenine and nicotinamide bases presumably because of the positive charge on the bases. However, both these bases stack reasonably well intermolecularly (Figs. 2 and 3), with stacking distances ranging from 2.82 to 3.96 Å due to a slight tilt between the bases. The water molecule W2 stacks on top of C-4N forming a half-open water sandwich (*11*). The formation of sandwiches and half-sandwiches of water molecules between nucleic acid bases has been reported (*11, 12*). The occurrence of such a sandwich structure in $NAD^+$ gives additional support to the contention that a water molecule will be able to stack on or intercalate between nucleic acid bases, especially if the bases are electron deficient. The reactive center of the nicotinamide molecule, namely C-4N, is sandwiched between the water molecule W2 and the positively charged N-1A of adenine. The relative disposition of W2 with respect to the nicotinamide ring in $NAD^+$ is somewhat similar to that of W439 in the

nicotinamide-binding pocket of dihydrofolate reductase (*13*).

A comparison of the three-dimensional conformation of $NAD^+$ as found in this structure with those of the $Li^+$-$NAD^+$ complex and $NAD^+$ complexed to several dehydrogenases (see Table 1 and Fig. 1) reveals a conformational difference between free and bound $NAD^+$ molecules. In the free-acid form, the molecule shows a compact shape in spite of the positive charges on the two bases; the adenine and nicotinamide rings are parallel and their centers are approximately 9.6 Å apart. In the $Li^+$ complex, the binding of $Li^+$ to two unesterified oxygen atoms of adjacent phosphates PA and PN causes eclipsed conformation across the virtual PN . . . PA bond and alters the backbone conformation. At physiological *p*H, however, there is no charge on adenine, but a hydrogen bond from a side chain to N-1 of adenine in the hydrophobic pocket of the enzyme will enable $NAD^+$ to mimic the electronic state of the molecule in the free-acid form. For example, in lactate dehydrogenase the OH of tyrosine-85 is hydrogen-bonded to N-1 of adenine (*14*). Similarly, the effect of $Li^+$ ion may be replaced in the enzyme by positively charged amino acid side chains. Although it is possible for enzyme-bound $NAD^+$ to assume a conformation similar to that of the free-acid or the $Li^+$ complex, the actual conformation of bound $NAD^+$ in the known complexes (Table 1) differs in an important way from the free-acid or $Li^+$ complex. One or both of the nucleotides in $NAD^+$ is not in the usually observed standard low-energy form (*9*) but takes up the higher energy nonstandard $g^-$ or *trans* conformation across the C-4′–C-5′ bond. Such higher energy conformations also persist when the coenzyme NADH is bound to glycogen phosphorylase b (*15*). Although $NAD^+$ is bound to dehydrogenases in an extended form and the interaction of the adenosine moiety with the enzyme is similar, the details of the coenzyme conformation in individual coenzyme-enzyme complexes differ (Table 1), thereby reflecting both the geometric similarity and the evolutionary relation of nucleotide binding sites (*16, 17*). In spite of these individual variations, there seems to be no ambivalence in the way in which the A dehydrogenases bind the nicotinamide with *anti* conformation and B dehydrogenases with *syn* conformation (*4, 18*). This difference cannot be due to any energy barrier for *syn-anti* conversion, since the driving force to form the enzyme-substrate complex is sufficient to overcome the *syn-anti* potential barrier of even 8-brominated coenzymes (*19*). Besides, the lack of a keto group on C-2 in nicotinamide as compared to its presence in pyrimidines makes this barrier for nicotinamide smaller than for pyrimidines. There have been many attempts to discern a pattern in the different stereo preferences of A and B dehydrogenases (*18, 20*), and many rules have been proposed but none has a clear mechanistic basis. A correlation between these stereochemical preferences and the thermodynamic stability of their substrates has been found, and it has been suggested that the conformation of the cofactor be chosen so that its reducing power matches the reducibility of the substrate (*21*). It is remarkable that the conformational flexibility of the nicotinamide coenzymes is used on the one hand to match the catalytic site of A and B dehydrogenases and on the other hand to adjust to the divergence of nucleotide binding sites and their evolution.

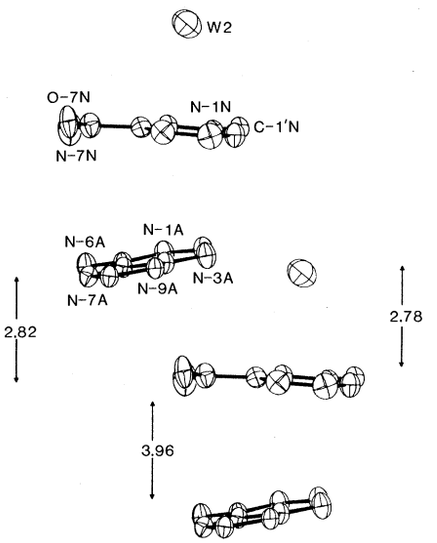


Fig. 2. Edge view of the stacking of the water molecule on the nicotinamide base and the nicotinamide sandwiched between adenine and water; the numbers indicate stacking distances (in angstroms).

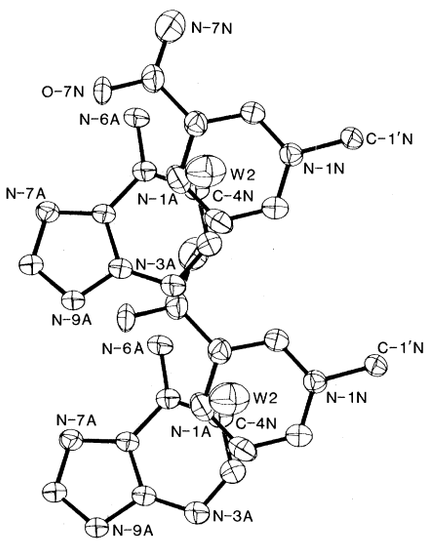


Fig. 3. Top view of the stacking of the bases and the nicotinamide sandwich.

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