PROTEINS: Structure, Function, and Genetics 28:10–28 (1997)

RESEARCH ARTICLES

NADP-Dependent Enzymes. I: Conserved Stereochemistry of Cofactor Binding

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ABSTRACT **The ubiquitous redox cofactors nicotinamide adenine dinucleotides [NAD and NADP] are very similar molecules, despite their participation in substantially different biochemical processes. NADP differs from NAD in only the presence of an additional phosphate group esterified to the 2′-hydroxyl group of the ribose at the adenine end and yet NADP is confined with few exceptions to the reactions of reductive biosynthesis, whereas NAD is used almost exclusively in oxidative degradations. The discrimination between NAD and NADP is therefore an impressive example of the power of molecular recognition by proteins. The many known tertiary structures of NADP complexes affords the possibility for an analysis of their discrimination. A systematic analysis of several crystal structures of NAD(P)-protein complexes show that: 1) the NADP coenzymes are more flexible in conformation than those of NAD; 2) although the protein-cofactor interactions are largely conserved in the NAD complexes, they are quite variable in those of NADP; and 3) in both cases the pocket around the nicotinamide moiety is substrate dependent. The conserved and variable interactions between protein and cofactors in the respective binding pockets are reported in detail. Discrimination between NAD and NADP is essentially a consequence of the overall pocket and not of a few residues. A clear fingerprint in NAD complexes is a carboxylate side chain that chelates the diol group at the ribose near the adenine, whereas in NADP complexes an arginine side chain faces the adenine plane and interacts with the phosphomonoester. The latter type of interaction might be a general feature of recognition of nucleotides by proteins. Other features such as strand-like hydrogen bonding between the NADP diphosphate moeties and the protein are also significant. The NADP binding pocket properties should prove useful in protein engineering and design. Proteins 28:10–28, 1997**

Key words: nicotinamide adenine dinucleotide; nicotinamide adenine dinucleotide phosphate; dinucleotide binding pockets; molecular recognition; protein structures

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate [NADP] are ubiquitous coenzymes in the biological redox reactions of all living systems. In the presence of the relevant enzyme and substrate they formally transfer a hydride ion from or to the carbon atom in position 4 of the nicotine ring in a stereospecific way. Despite their great structural similarity with NADP differing from NAD only by the presence of an additional phosphate group esterified to the 2′-hydroxyl group of the ribose at the adenine end, NAD and NADP differ quite substantially in their biochemistry. Although NAD is used almost exclusively in oxidative degradations that yield ATP and thus behaves as an oxidant, NADP is confined with few exceptions to the reactions of reductive biosynthesis and behaves therefore as a reductant.

The fact that such a small structural difference imparts such a large biochemical difference is of course surprising and yet provides one of the most striking examples of the power of the molecular recognition in biological chemistry. In only a few cases do NAD or NADP-dependent enzymes not display a strong preference for one of the two cofactors. The best known is given by glutamate dehydrogenases, a family of enzymes whose cofactor specificity ranges from strict NADP to strict NAD and also

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Received 8 July 1996; Accepted 18 October 1996

includes examples in which either cofactor may be used.[1] In recent years, a few successful attempts at protein engineering have been reported in which the coenzyme specificity has been inverted by mutating a few residues that play an obvious role in NAD/NADP discrimination.[2–4] Nevertheless, a general explanation for such molecular discrimination by proteins is missing, despite several works devoted to the analysis and several crystal structure comparisons of NADP binding proteins.

An early rationalization[5] suggested that the sequence GXGXXG or GXGXXA (where X is any amino acid) determines the interaction between the protein and the diphosphate moiety of the NADP cofactor; such a sequence is present in the loop between the first β strand and a subsequent α helix of the NADP binding domain. At the end of the second and subsequent parallel β strand, a carboxylic side chain (aspartate or glutamate) interacts with the diol group of the ribose located at the adenine end of the NAD coenzyme but it is absent in NADP binding pockets. A later investigation by Baker et al.[6] confirmed the previous results but they found some exceptions to the GXGXXG/A rule, specifically in the case of malate dehydrogenase. Moreover, the above authors noted that the GXGXXG/A fragment interacts with the diol group of the ribose near the adenine, either directly or indirectly (via the carboxylate side chain) and depending on the last residue of the GXGXXG/A protein fragment. Nevertheless, many obscure points still remained, such as the importance of the interaction between the carboxylate side chain and the diol group of the ribose near the adenine. Baker et al.[6] actually observed that a Glu residue is found in the dual specificity glutamate dehydrogenase. Few studies on molecular discrimination between NAD and NADP have yielded a reasonable explanation. In comparing alcohol dehydrogenase (a NAD-dependent enzyme) and quinone oxidoreductase [NADP dependent], Lesk[7] noted that the space occupied by an aspartate side chain in the NAD complex is occupied by the 2′-phosphomonoester in the NADP complex where a glycine substitutes for the aspartate. However, this complementary mutuation is not observable in 6-phosphogluconate dehydrogenase, another NADP-dependent enzyme, in which NADP is accommodated in a completely different way. The aspartate side chain of alcohol dehydrogenase is substituted by an asparagine able to form hydrogen bonds with both the 2′-phosphomonoester and the adenine ribose hydroxyl group.

In this study we report a systematic analysis of the known crystal structures of NAD and NADP-bound proteins. A nonredundant set of 32 structures has been selected from the Protein Data Bank,[8] representing a total of 19 enzymes: 21 entries are NAD complexes and 11 are NADP (Fig. 1 and Table I). Such a data set is the largest ever examined and is considerably updated especially since 27 of the 32 (85%) entries have been deposited in the Protein Data Bank during the last 4 years. Both the cofactor conformations and the binding site stereochemistries are presented and compared. The results were examined from two aspects: properties that allow molecular discrimination of NAD and NADP and evolutionary development of the NADP binding enzymes. The first is exhaustively investigated here, whereas the latter is developed in the another study and is based in part on the results given here.

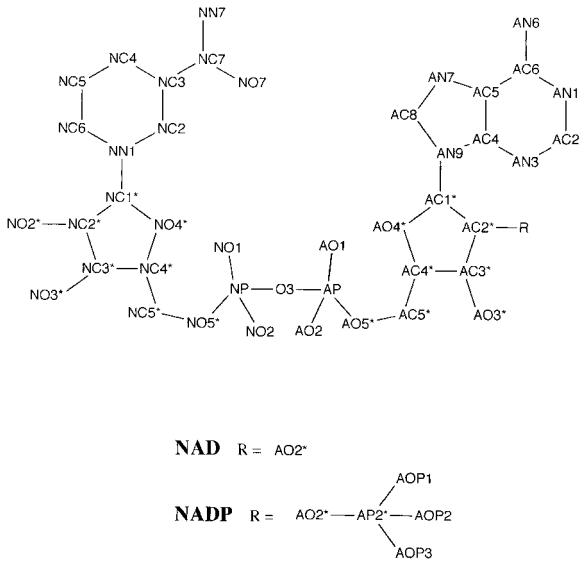


Fig. 1. Numbering scheme for the atoms in NAD and NADP molecules. The standard nomenclature of the Protein Data Bank[8] is adopted. The letter A or N before the atomic chemical symbol (C, O, N, or P) indicates adenine or nicotinamide nucleotide, respectively.

Such studies are only possible if the number of experimental structures is sufficiently large. The NADP binding enzymes, besides their intrinsic biological relevance, are probably the most studied systems in which a common cofactor is found in many crystal structures. Other systems such as heme or metal binding proteins, even if well studied, are less interesting because the former permanently bind the cofactor, contrary to most enzymes, and the latter bind a single atom as coenzyme with attendant poorer stereochemistry relative to that involving an organic molecule. The principles concerning molecular recognition of NAD and NADP represent a canonical system and will probably point to general strategies utilized in many other biological systems.

RESULTS

Conformation of NADP cofactors

The chemically equivalent atoms in all possible pairs of the 32 NADP coenzymes considered in this study were spatially superposed. The terminal oxygen atoms of the diphosphate moiety (AO1, AO2, NO1, and NO2) and the atoms within the monophos-

TABLE I. Listing of Examined NADP Tertiary Structural Protein Complexes

Protein data bank	Enzyme	Source	Resolution (Å)
NAD-containing structures			
1BMD+	malate dehydrogenase	*Thermus flavus*	1.90
1DHR	dihydropteridine reductase	*Rattus norvegicus*	2.30
1EMD	malate dehydrogenase	*Escherichia coli*	1.90
1GD1+	D-glyceraldehyde-phosphate dehydrogenase	*Bacillus stearothermophilus*	1.80
1GEU+	glutathione reductase	*Escherichia coli*	2.20
1GGA+	D-glyceraldehyde-phosphate dehydrogenase	*Trypanosoma brucei brucei, glycosoma*	3.20
1HDG+	D-glyceraldehyde-phosphate dehydrogenase	*Thermotoga maritima*	2.50
1HDR	dihydropteridine reductase	*Homo sapiens*	2.50
1HDX+	alcohol dehydrogenase	*Homo sapiens*	2.50
1HLP+	malate dehydrogenase	*Haloarcula marismortui*	3.20
1LDM	lactate dehydrogenase	*Squalus acanthias*	2.10
1LDN+	lactate dehydrogenase	*Bacillus stearothermophilus*	2.50
1LLD+	lactate dehydrogenase	*Bifidobacterium longum*	2.00
1PSD+	D-3-phosphoglycerate dehydrogenase	*Escherichia coli*	2.75
1LVL	dihydrolipoamide dehydrogenase	*Pseudomonas putida*	2.45
2HSD+	3-α,20-β-hydroxysteroid dehydrogenase	*Streptomyces hydrogenans*	2.64
2NAD+	formate dehydrogenase	*Methylotrophic bacterium pseudomonas*	2.00
2NPX	NADH peroxidase	*Stroptococcus faecalis*	2.40
2OHX+	alcohol dehydrogenase	*Equus caballus*	1.80
4MDH+	malate dehydrogenase	*Sus scrofa*	2.50
9LDT+	lactate dehydrogenase	*Sus scrofa*	2.00
NADP-containing structures			
1ADS	aldose reductase	*Homo sapiens*	1.60
1CAF	catalase	*Proteus mirabilis*	3.10
1DLR	dihydrofolate reductase	*Homo sapiens*	2.30
1DRH	dihydrofolate reductase	*Escherichia coli*	2.30
1IKB	isocitrate dehydrogenase	*Escherichia coli*	2.50
1PGO	6-phosphogluconate dehydrogenase	*Escherichia coli*	2.50
1QOR+	quinone oxidoreductase	*Escherichia coli*	2.20
1TCS	trichosanthin	*Trichosanthes kirilowiimaxim*	1.70
1TYP+	trypanothione reductase	*Crithida fasciculata*	2.80
3DFR	dihydrofolate reductase	*Lactobacillus casei*	1.70
8DFR	dihydrofolate reductase	*Gallus gallus*	1.70

In these proteins, where the crystallographic asymmetric unit contains more than one subunit, only the first monomer was considered for analysis. The structural differences among the related subunits were limited: the root-mean-square distance between all spatially equivalenced Cα atoms ranged from 0.0 to 1.5 Å with mean value at 0.38 Å, whereas the root-mean-square distance between superposed cofactor atoms ranged between 0.0 and 1.1 Å with a mean value of 0.32 Å.

phoester group [AP2*(AOP1)(AOP2)(AOP3)] were disregarded because of possible inconsistency in their labeling. The resulting set of 40 atom-based root-mean-square distance values was used in cluster analysis, results of which are summarized in Figure 2.

The 13 classes appear plausible. Among them, 8 contain only one structure, 1 has two cofactors, 2 with three, 1 with four, and 1 with. Cluster 1, the biggest, contains malate, lactate, alcohol, formate and D-3-phosphoglycerate dehydrogenase. Dihdrofolate reductases constitute the four-membered cluster 6. Among the two groups with three entries each, one contains only D-glyceraldehyde-phosphate dehydrogenases (cluster 2), whereas the other comprises dihydropteridine reductase and 3-α-20-β-hydroxysteroid dehydrogenase (cluster 3). Cluster 7 is formed by glutathione reductase and NADH peroxidase.

Some groups are expected. The four dihydrofolate reductases (1DLR, 1DRH, 3DFR, and 8DFR) cluster reflect similar biochemistry. The similarity between the conformations of the NADs bound to alcohol (1HDX and 2OHX) and lactate (1LDM, 1LDN, 1LLD, and 9LDT) dehydrogenases clearly resemble the close similarity among the folds of these enzymes.[9–10] Similar arguments can be made for dihydropteridine reductases (1DHR and 1HDR) and 3-α-20-β-hydroxysteroid dehydrogenase (2HSD).[11] However, it is quite surprising that 13 clusters are formed by only 32 entries, indicating a great conformational flexibility of NADP. This suggests that their ubiquity among different organisms and biochemical systems is a result of their flexibility. It is also quite surprising that NAD and NADP never cluster together and that NADP shows a bigger conformational freedom than NAD.

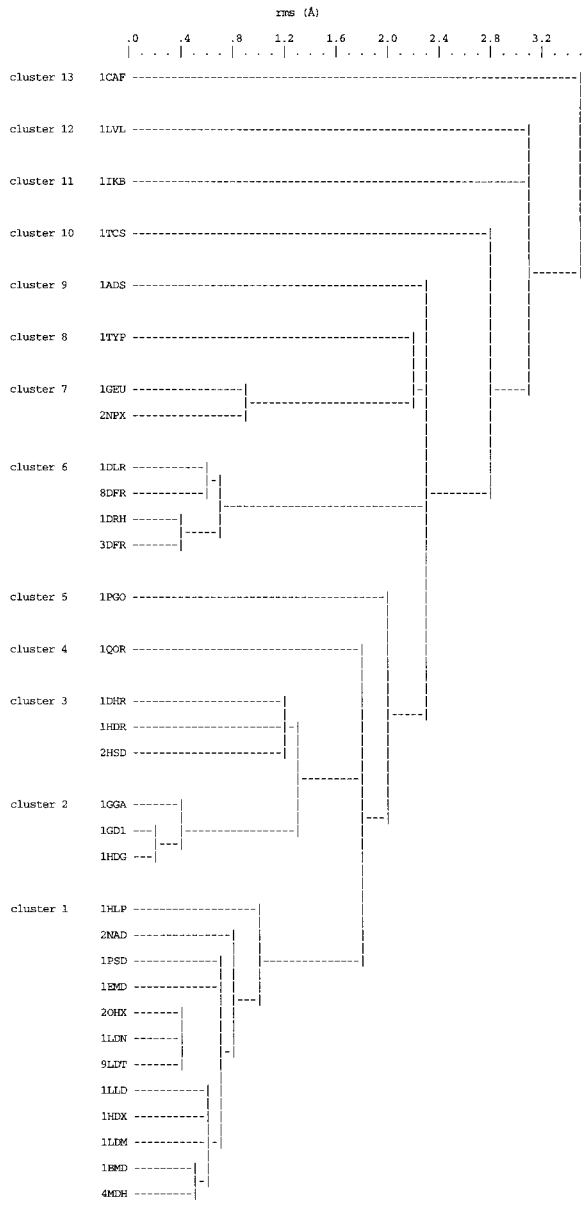


Fig. 2. Graph describing the cluster analysis on the NADP conformations. The cluster analysis is performed by a hierarchical agglomerative algorithm coupled with a single linkage similarity criterion. The pattern matrix elements are the root-mean-square distance values between equivalent atomic pairs obtained by superposing the NADP molecules.

Figure 3 shows NADP conformations for each of the 13 clusters. Table II reports selected structural parameters that characterize each group.

Among the stereochemical descriptors that can differentiate the 13 clusters, only a few torsions and interatomic distances between nonbonded atoms need be considered. In fact, many bond distances and angles are drastically biased by restraints in crystallographic refinements. Also, the nonplanarity of the nicotinamide moiety in the reduced cofactors cannot be definitely observed in crystal structures at medium and low resolution, and restraints on this stereochemical feature have been variously imposed by different authors.[12] Even the torsions within the riboses can be disregarded because they too are affected by crystallographic restraints.[13] The refined ribose puckering can be considered a consequence of the spatial arrangement of the ribose substituents.

The diphosphate fragment appears highly flexible. Under the rough assumption that the local conformation is a rotamer if it does not deviate more than 30° from the minimum energy value (60, 180, and −60° as a first approximation in the case of the diphosphate chain), it follows that many nonrotamers are present. Some of them are not energetically very unfavorable, such as the torsion AC4*-AC5*-AO5*-AP in clusters 2, 3, 4, 5, 8, and 10, which corresponds to an eclipsed conformation of AP toward a hydrogen atom. In other cases the deviation from the minimum energy is more problematic; e.g., the same torsion in cluster 11 corresponds to a nearly eclipsed conformation of AP toward AC4* and the ribose ring. Nevertheless, the casual diphosphate stereochemistry does not imply strong modification of the overall shape of the diphosphate itself. The distance between the terminal carbon atoms of the diphosphate group (AC4*-NC4*) does not assume completely random values: a group of clusters (1, 2, 3, 4, and 11) shows values around 6–7 Å and another (clusters 5, 6, 7, 8, 9, 10, and 12) shows values around 8–9 Å. Only the NADP of 1CAF (cluster 13) is very compressed and drastically different from the others. These observations imply that different sets of dihedral angles have been used to obtain similar diphosphate shapes and suggest that the modeling of the diphosphate torsions is not always locally very accurate.

More significant is the orientation of the diphosphate with respect to both ribose rings, which can be monitored by the NO5*-NC5*-NC4*-NO4* and the AO4*-AC4*-AC5*-AO5* torsions. With few exceptions, both dihedral angles are within the rough limits specified above in a minimum energy conformation. However, the possible combinations of torsional values are quite numerous (Table 2).

Another discriminant among the NADP conformations depends on the reciprocal orientations of the adenine and nicotinamide moieties with respect to their neighboring ribose rings, which can be monitored by the AO4*-AC1*-AN9-AC8 and NO4*-NC1*-NN1-NC2 torsions. In the first case, most entries have values of the AO4*-AC1*-AN9-AC8 torsion near −70° (clusters 1, 2, 3, 5, 7, 8, 9, 12, and 13). Concerning the orientation of the nicotine with respect to the ribose, five clusters have the NO4*-NC1*-NN1-NC2 torsion approaching 120° (clusters 1, 4, 5, 6, and 9) and three (2, 3, 13) have the same torsion near −60°. The latter groups have therefore supplementary values relative to the NO4*-NC1*-NN1-NC2 torsions and such a difference reflects the

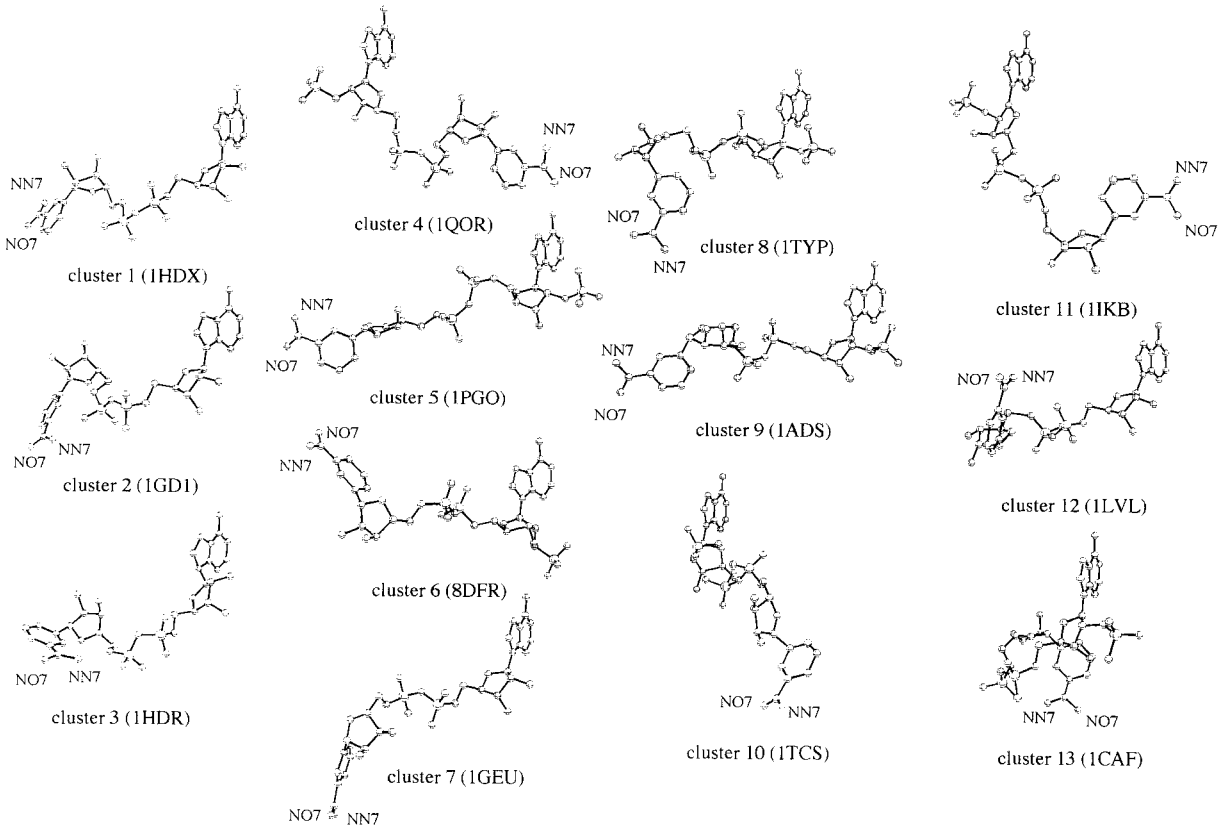


Fig. 3. View of various NADP conformations representative of the 13 groups detected by cluster analysis (Fig. 2). Atomic labels are indicated for the NN7 and NO7 atoms to show the orientations of the pyridine and carboxiamide groups.

well known enzyme stereospecificities for nicotinamide nucleotides;[14] i.e., clusters 1, 4, 5, 6, and 9 have A stereospecificity (in the sense that the hydride transfer takes place on the A face of the nicotine), whereas clusters 2, 3, and 13 have B stereospecificity. Nevertheless, values intermediate between 120° and −60° are observed and in these cases the stereospecificity of the hydride transfer may apparently be either A or B.

The orientation of the carboxyamide group on the nicotinamide ring can be used to group the NADP molecules. The NC2-NC3-NC7-NN7 torsion can assume only two values, which are 0 and 180° in the oxidized molecules in which the nicotinium ring is aromatic and planar. Values near 0 or 180° are observed in the examined data set (see Table 2). In nine clusters (1, 2, 3, 4, 5, 6, 9, 12, and 13) NC2-NC3-NC7-NN7 approaches 0°, whereas in the other four (7, 8, 10, and 11) it is near 180°.

Other quantities often considered to describe the stereochemistry of the NADP cofactors are also listed in Table II. Many authors have noted that nicotinamide and adenine tend to be perpendicular and separated by ~15 Å.[15] The angles formed by the least-squares planes between adenine and nicotinamide are actually quite variable, although they tend toward 90°. The distances between the centroids of the adenine and nicotinamide generally approach 15 Å, although values much lower (cluster 13) or higher (cluster 5) have been observed. As noted by Stoddard et al.,[15] the NADP molecules generally have an extended conformation drastically different from that found in the crystal structure of unbound NAD^+ in the free acid and lithium salt forms.[16–17]

NAD binding pocket characteristics

The pockets around the NADP cofactors were defined as all the enzyme and solvent atoms within a distance of 4.5 Å from any of the cofactor atoms. For the sake of clarity, results concerning the binding sites will be presented separately for the NAD and the NADP cofactors.

Figure 4 shows the results obtained by superposing the NAD molecules (over that of 1HDX) and projecting into the same space the atoms forming all the pockets.

The most relevant feature appearing is the different distribution of pocket-forming atoms around the nicotine and adenine ends of NAD. Although around the nicotine a continuum of atoms is observed,

TABLE II. Mean Values Computed Over the Members of Each Cluster of Selected Conformational Features of the NAD and NADP Molecules With Estimated Standard Deviations in Parentheses

Torsions (deg)											
A	NC2	NO4*	AO4*	AO4*	AC4*	AC5*	AO5*	AP	O3	NP	NO5*
B	NC3	NC1*	AC1*	AC4*	AC5*	AO5*	AP	O3	NP	NO5*	NC5*
C	NC7	NN1	AN9	AC5*	AO5*	AP	O3	NP	NO5*	NC5*	NC4*
D	NN7	NC2	AC8	AO5*	AP	O3	NP	NO5*	NC5*	NC4*	NO4*
Cluster 1	−10 (1.0)	110 (1.0)	−78 (1.0)	171 (0.9)	−150 (0.9)	−66 (0.8)	−83 (0.7)	156 (0.7)	−68 (0.7)	178 (1.0)	72 (0.8)
Cluster 2	32 (1.0)	−75 (1.0)	−75 (1.0)	−165 (1.0)	−146 (1.0)	−82 (1.0)	−79 (1.0)	153 (1.0)	−71 (1.0)	−164 (1.0)	56 (1.0)
Cluster 3	13 (0.3)	−65 (1.0)	−77 (0.9)	169 (0.8)	−133 (1.0)	−72 (1.0)	−78 (0.9)	156 (1.0)	−72 (0.9)	161 (1.0)	36 (0.9)
Cluster 4	2 (1.0)	118 (1.0)	130 (1.0)	−169 (1.0)	93 (1.0)	81 (1.0)	−173 (1.0)	113 (1.0)	−58 (1.0)	171 (1.0)	71 (1.0)
Cluster 5	25 (1.0)	129 (1.0)	−58 (1.0)	−50 (1.0)	−112 (1.0)	70 (1.0)	−179 (1.0)	−87 (1.0)	−169 (1.0)	−98 (1.0)	−159 (1.0)
Cluster 6	−7 (1.0)	118 (1.0)	−18 (1.0)	−78 (1.0)	156 (1.0)	81 (1.0)	−153 (1.0)	−77 (1.0)	−63 (0.9)	−133 (1.0)	−64 (1.0)
Cluster 7	167 (1.0)	169 (1.0)	−71 (1.0)	−171 (1.0)	−170 (1.0)	−53 (1.0)	−91 (1.0)	−84 (1.0)	−58 (1.0)	152 (1.0)	67 (1.0)
Cluster 8	180 (1.0)	168 (1.0)	−77 (1.0)	167 (1.0)	−117 (1.0)	−160 (1.0)	−7 (1.0)	−179 (1.0)	−72 (1.0)	−126 (1.0)	−89 (1.0)
Cluster 9	−6 (1.0)	131 (1.0)	−74 (1.0)	−63 (1.0)	−177 (1.0)	64 (1.0)	−149 (1.0)	−23 (1.0)	86 (1.0)	101 (1.0)	59 (1.0)
Cluster 10	162 (1.0)	176 (1.0)	98 (1.0)	−25 (1.0)	−119 (1.0)	−144 (1.0)	−91 (1.0)	−142 (1.0)	145 (1.0)	−162 (1.0)	−11 (1.0)
Cluster 11	−176 (1.0)	−143 (1.0)	172 (1.0)	120 (1.0)	−28 (1.0)	−16 (1.0)	20 (1.0)	−7 (1.0)	130 (1.0)	158 (1.0)	109 (1.0)
Cluster 12	1 (1.0)	−26 (1.0)	−75 (1.0)	179 (1.0)	−162 (1.0)	−104 (1.0)	49 (1.0)	73 (1.0)	86 (1.0)	−26 (1.0)	−60 (1.0)
Cluster 13	3 (1.0)	−56 (1.0)	−96 (1.0)	−174 (1.0)	−162 (1.0)	−74 (1.0)	−72 (1.0)	110 (1.0)	57 (1.0)	−159 (1.0)	50 (1.0)

Angles (deg) formed by least-squares planes between (a) adenine (A) and nicotinamide (N), (b) adenine and ribose A, (c) adenine and ribose N, (d) nicotinamide and ribose A, (e) nicotinamide and ribose N, and (f) ribose A and ribose N

	(a)	(b)	(c)	(d)	(e)	(f)
Cluster 1	85 (1.0)	89 (1.0)	88 (0.9)	16 (0.8)	80 (1.0)	82 (1.0)
Cluster 2	79 (1.0)	84 (1.0)	107 (0.8)	12 (1.0)	89 (1.0)	88 (1.0)
Cluster 3	94 (0.9)	96 (1.0)	107 (0.7)	91 (0.8)	82 (1.0)	95 (1.0)
Cluster 4	70 (1.0)	78 (1.0)	74 (1.0)	23 (1.0)	97 (1.0)	79 (1.0)
Cluster 5	55 (1.0)	82 (1.0)	99 (1.0)	50 (1.0)	77 (1.0)	27 (1.0)
Cluster 6	110 (0.8)	97 (1.0)	88 (0.9)	38 (1.0)	86 (1.0)	83 (0.9)
Cluster 7	24 (1.0)	80 (1.0)	89 (1.0)	57 (1.0)	86 (1.0)	88 (0.6)
Cluster 8	72 (1.0)	102 (1.0)	112 (1.0)	66 (1.0)	75 (1.0)	11 (1.0)
Cluster 9	126 (1.0)	97 (1.0)	111 (1.0)	108 (1.0)	38 (1.0)	144 (1.0)
Cluster 10	69 (1.0)	56 (1.0)	21 (1.0)	105 (1.0)	89 (1.0)	40 (1.0)
Cluster 11	65 (1.0)	60 (1.0)	79 (1.0)	114 (1.0)	92 (1.0)	46 (1.0)
Cluster 12	25 (1.0)	78 (1.0)	102 (1.0)	55 (1.0)	80 (1.0)	27 (1.0)
Cluster 13	105 (1.0)	57 (1.0)	103 (1.0)	58 (1.0)	81 (1.0)	122 (1.0)

Distances (Å) (a) between NC4* and AC4* and (b) the center of mass of the nicotinamide and adenine

	(a)	(b)
Cluster 1	6.6 (0.3)	15.1 (0.5)
Cluster 2	7.0 (0.1)	15.0 (0.4)
Cluster 3	6.6 (0.7)	14.4 (0.2)
Cluster 4	6.1 (0.0)	14.1 (0.0)
Cluster 5	8.3 (0.0)	18.1 (0.0)
Cluster 6	8.9 (0.0)	15.1 (0.6)
Cluster 7	8.5 (0.0)	16.6 (0.0)
Cluster 8	8.5 (0.0)	15.0 (0.0)
Cluster 9	8.2 (0.0)	16.5 (0.0)
Cluster 10	9.6 (0.0)	17.8 (0.0)
Cluster 11	6.7 (0.0)	14.8 (0.0)
Cluster 12	7.9 (0.0)	14.1 (0.0)
Cluster 13	4.9 (0.0)	8.0 (0.0)

The torsions are referred to in the form A/B/C/D and are the angles between the normals to the planes defined by the A, B, and C atoms and the B, C, and D atoms. The mean values of the torsions and the angles between least-squares planes are calculated by circular statistics techniques because in both cases the individual values are periodic; in these cases in parentheses the dispersion-estimated values are reported that are close to 1.0 as the sample is little dispersed, whereas values near 0.0 indicate a randomly distributed sample.

around the adenine clustering is apparent. This result is not surprising. The chemical activity of NAD is located at the nicotine ring and consists of a hydride anion transfer to or from the NC4 carbon atom. Both the shape and the physicochemical features of the pocket around the nicotine moiety are substrate dependent and are partially responsible for the substrate orientation. The high variability of possible substrates correspondingly yields the variable nicotine pocket. In contrast, the substantial conservation of the pocket stereochemistry around the adenine moiety indicates that the ADP atoms interact with the protein independently of the substrate specificity.

Figure 4 shows that there are primarily four regions in which the atoms forming the pocket cluster together. An extended pocket conformation is parallel to the diphosphate moiety of the NAD molecule. A group of atoms is located in the proximity of the diol group of the ADP ribose and the A face

Fig. 4. Superposition of the NAD molecules (brown stick model) and of the atoms defining their pockets (red stick model = protein atoms; grey spheres = water molecules). The NAD molecules are oriented with the adenine moieties on the right and the nicotinamide on the left. The pocket atoms generally appear uniformly distributed about the nicotinamide moieties. In contrast, in four zones around the other half of the cofactors groups of atoms cluster within the binding pockets and are indicated by captions and arrows. A first cluster appears as an extended conformation nearly parallel to the cofactor diphosphate moiety. A second is in front of the A face of the adenine. A third cluster before the B face of the adenine is clearly divided into two subclusters, one near the upper edge of the adenine and the other near the lower edge of the adenine and is also close to the ribose near the nicotinamide moiety. A fourth cluster consists of water molecules near the AN6 and AN1 nitrogen atoms of the adenine.

of the adenine. A group of atoms is concentrated in front of the B face of the adenine. A group of water molecules clusters near the AN6 and AN1 atoms of the adenine moiety.

The protein fragment nearly parallel to the diphosphate group consists of six or seven residues (positions from S1 to S7). Three residues (positions S4, S5, and S6) interact with the diphosphate oxygen atoms through hydrogen bonds with main chain atoms as shown in Figure 5. The residue at site S4 is invariably a glycine and that in position S6 is always a hydrophobic amino acid, whereas the residue at site S5 is quite variable (Table III). The other residues, preceding and after the S4–S6 triplet, interact with other atoms of the NAD molecule and some of their main characteristics have already been noted and discussed.[5] The residue at site S5 forms a bifurcated hydrogen bond with both of the NAD phosphate groups. Residue S6 forms a hydrogen bond with a phosphate oxygen atom also bonded to the main chain of S5. S4, invariably a glycine, appears to interact with the diphosphate group through an unusual C-H . . . O hydrogen bond (Cα . . . AO1 and Cα . . . AO5*), although this stereochemical arrangement could be simply a consequence of the local packing. Though the existence and importance of these exotic hydrogen bonds is controversial,[18–21] a similar hydrogen bond arrangement has been noted by Derewenda et al.[22] in β sheets.

The polypeptide fragment rich in glycines and interacting with the NAD diphosphate group is within a tight loop connecting a β strand and an α helix nearly at the beginning of the α helix. In all the 21 NAD-bound protein structures examined here a more or less canonical Rossmann fold[23] has been observed. It consists of six stranded parallel β sheet with α helices connecting successive β strands and lying on both sides of the sheet. In all cases the polypeptide fragment interacting with the diphosphate group is within the loop between the first β strand and the first α helix. The backbone conformational freedom required for the turn as well as for a nonobstructed pocket are probable explanations for the glycine-rich character of this fragment. The presence of a hydrophobic residue in position S6 is probably due to its side chain direction toward the nicotine ring with conserved apolar environment.

A second conserved NAD pocket region faces the diol of the ADP ribose and the adenine A face. Three residues (S8, S9, and S10) contribute to this region of the pocket (Fig. 6 and Table IV). The S9 residue, an aspartate (more frequently observed) or a glutamate (less frequently observed), chelates the two oxygen atoms of the ADP ribose. The successive residue

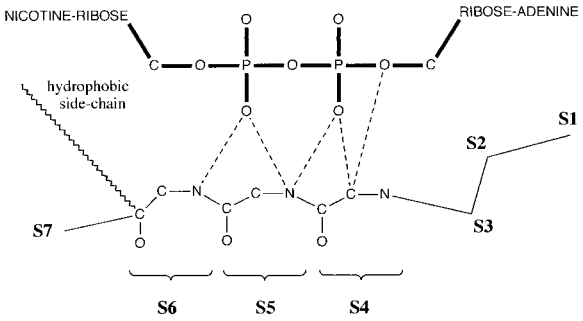


Fig. 5. Schematic representation of the interactions between the protein and the diphosphate moiety of the NAD molecule. A group of six or seven residues are indicated according to the sites they occupy (S1–S7; residue in position S2 is not always present). They are in a loop between an antiparallel β strand and α helix and just preceed the α helix. The protein segment is glycine rich with S1, S4, and S7 nearly always glycine. The other residues are quite variable but at site S6 an apolar side chain is conserved and directed toward the nicotinamide moiety. The diphosphate terminal oxygen atoms are hydrogen bonded to the main chain nitrogen atoms of residues S5 and S6; the Cα atom of residue S4 is close to oxygen atoms of the diphosphate according to van der Waals atomic radii criteria and perhaps makes an attractive interaction of the type C-H . . . O. Residues S1–S3 are hydrogen bonded with a protein segment far in the sequence from S1–S7, which interacts with the adenine and its neighbor ribose.

(S10) faces the A face of the adenine, whereas the preceding (S8) interacts through a hydrogen bond (C = O . . . H–N) with the S1 residue, a constituent of the NAD diphosphate interacting fragment (Fig. 7). All of the three residues are in a loop connecting the second β strand and the following α helix of the Rossmann fold. The hydrogen bond between the residues in positions S8 and S1 causes the formation of a type of chair on which the adenine sits. Because this hydrogen bond involves only main chain atoms, no strong conservation of the S8 residue is observed, whereas S1 is very conserved (almost always a glycine with only one alanine exception), again because of stereochemical reasons. The two hydrogen bonds between the diol group of the ADP ribose and the residue in position S9 probably contribute strongly to the thermodynamics of NAD binding. Moreover, the carboxylate side chain moiety of the S9 residue is hydrogen bonded to the main chain nitrogen atoms of residues S2 and S3. Although the residue in position S10 always directs its side chain toward the adenine A face, it is not well conserved. Either apolar or polar amino acids can be found in this position, although for polar side chains (e.g., arginine) the segment of the side chain in front of the A face is always apolar. Therefore, a hydrophobic interaction with the delocalized π adenine takes place.system of the adenine takes place.

In cases of identical residues at site S10, the side chain conformations are often quite similar. For example, in 9 of 21 occupations of S10 by an isoleucine, the torsions N-CA-CB-CG1, N-CA-CB-CG2, and CA-CB-CG1-CD1 are always within 10° of the respective rotameric values of −60°, 60°, and 180° with only one exception (1PSD). Again, for 5 of the 21 sites occupied by a valine the N-CA-CB-CG1 and N-CA-CB-CG2 torsions are within 20° of the respective 60° and −60° values with only one exception (2HSD). This suggests that the adenine-side chain interaction is strong and directional. This could be justified by the anisotropy of the delocalized π system of the adenine, whose rings approximately present alternating electronegative atoms (nitrogens and carbons). Such an interaction has been recently claimed as a C-H . . . π-electron hydrogen bond.[24] The projections of homologous side chains onto the plane of the adenine A face do not always superpose well (Fig. 8), thus weakening the contention of directionality in this interaction.

A third zone of clustered atoms within the NAD pockets is found in front of the adenine B face. There is not a single strong trend among the various structures; however, a common feature of all the complexes is that the atoms facing the B side of the adenine belong to residues that either directly or indirectly (i.e., via water molecules) also interact with the ribose of the nicotine end of the NAD (or in some cases with the phosphate group near this ribose). Fundamentally, two types of interactions are observed. The side chain of an apolar residue is directly in front of the B face or various side chains cooperate to cover the B face. These two major patterns are shown and summarized in Figure 9 and Table V. When more than one residue faces the B side (pattern 1), two residues (positions S11 and S12) projecting onto the lower part of the B face are nearly always glycines or alanines. Although the first residue projects its side chain (or in the case of glycine its aliphatic hydrogen atoms) toward the adenine, the second is primarily involved in hydrogen bonding via its main chain nitrogen atom with the endocyclic oxygen of the ribose. Moreover, in pattern 1 the side chains of two other residues (positions S13 and S14) are in front of the upper part of the B face. They may be either polar or apolar, although more frequently apolar, and do not show any particularly conserved electrostatic interaction with the adenine nitrogen atoms. The former residue pair is always located just after the fourth β strand of the canonical Rossmann fold. The second pair is always in the middle of the fourth α helix in the canonical Rossmann fold and they are always spaced by three residues as a consequence of the helical geometry.

Pattern 2 (Fig. 9) differs considerably from pattern 1. A single side chain of a residue located just after the fourth β strand in the canonical Rossmann fold is in front of the adenine B face (residue in position S15). It may be either polar or apolar, although in the first pattern only apolar atoms are directed toward the adenine plane. In all cases a glycine (site S16) is observed after this residue and allows the backbone direction to turn. In five of seven pattern 2 examples

TABLE III. Sequences of the Protein Fragments Interacting With the Diphosphate Moiety of the NAD Molecules

Protein data bank	Residue													
	S1		S2		S3		S4		S5		S6		S7	
1BMD	gly	10	ala	11	ala	12	gly	13	gln	14	ile	15	gly	16
1DHR	gly	13	gly	14	arg	15	gly	16	ala	17	leu	18	gly	19
1EMD	gly	7	ala	8	ala	9	gly	10	gly	11	ile	12	gly	13
1GD1	gly	7	—	—	phe	8	gly	9	arg	10	ile	11	gly	12
1GEU	gly	174	—	—	ala	175	gly	176	tyr	177	ile	178	gly	179
1GGA	gly	8	—	—	phe	9	gly	10	arg	11	ile	12	gly	13
1HDG	gly	7	—	—	phe	8	gly	9	arg	10	ile	11	gly	12
1HDR	gly	16	gly	17	arg	18	gly	19	ala	20	leu	21	gly	22
1HDX	gly	199	—	—	leu	200	gly	201	gly	202	val	203	gly	204
1HLP	ala	27	ala	27a	ala	28	gly	29	thr	30	val	31	gly	32
1LDM	gly	27	—	—	val	28	gly	29	ala	30	val	31	gly	32
1LDN	gly	27	—	—	ala	28	gly	29	phe	30	val	31	gly	32
1LLD	gly	14	—	—	ala	15	gly	16	ala	17	val	18	gly	19
1LVL	gly	178	—	—	gly	179	gly	180	tyr	181	ile	182	gly	183
1PSD	gly	158	—	—	tyr	159	gly	160	his	161	ile	162	gly	163
2HSD	gly	13	gly	14	ala	15	arg	16	gly	17	leu	18	gly	19
2NAD	ala	198	—	—	ala	199	gly	200	arg	201	ile	202	gly	203
2NPX	gly	156	—	—	ser	157	gly	158	tyr	159	ile	160	gly	161
2OHX	gly	199	—	—	leu	200	gly	201	gly	202	val	203	gly	204
4MDH	gly	10	ala	11	ala	12	gly	13	gln	14	ile	15	ala	16
9LDT	gly	28	—	—	val	29	gly	30	ala	31	val	32	gly	33

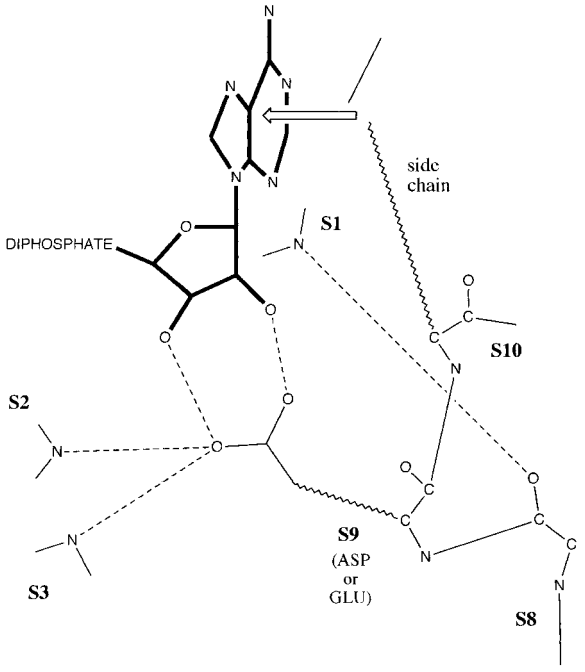


Fig. 6. Schematic representation of the interactions between the protein and the NAD A face of the adenine and its neighbor ribose. Three residues, referred to by sites they occupy (from S8 to S10), are considered. They are in a loop between an antiparallel β strand and α helix but just at the end of the β strand. The first residue (S8) is hydrogen bonded to residue S1 (see Fig. 5). The second (S9), usually an aspartate or less frequently a glutamate, forms a double hydrogen bond with the diol group of the ribose near the adenine and is in turn hydrogen bonded to the residues S2 and S3 (Fig. 5). The third (S10) directs its side chain toward the A face of the adenine. Residues S8 and S10 are little conserved, although the latter is frequently apolar.

TABLE IV. Sequences of the Protein Fragments Interacting With the A Face of the Adenine and the Adenine Ribose of the NAD Molecules

Protein data bank	Residue					
	S8		S9		S10	
1BMD	leu	40	glu	41	ile	42
1DHR	ile	36	asp	37	val	38
1EMD	tyr	33	asp	34	ile	35
1GD1	asn	31	asp	32	leu	33
1GEU	phe	196	glu	197	met	198
1GGA	val	36	asp	37	met	38
1HDG	asn	31	asp	32	leu	33
1HDR	val	39	asp	40	val	41
1HDX	val	222	asp	223	ile	224
1HLP	val	51	asp	52	ile	53
1LDM	val	51	asp	52	val	53
1LDN	ile	51	asp	52	ala	53
1LLD	glu	38	asp	39	ile	40
1LVL	val	200	glu	201	ala	202
1PSD	tyr	180	asp	181	ile	182
2HSD	ala	36	asp	37	val	38
2NAD	thr	220	asp	221	arg	222
2NPX	ile	178	asp	179	ile	180
2OHX	val	222	asp	223	ile	224
4MDH	leu	40	asp	41	ile	42
9LDT	val	52	asp	53	val	54

the residue (site S17) after the glycine, which can be either polar or apolar, although no specific and conserved electrostatic interactions appear, is located near the upper edge of the adenine. In other cases the backbone direction is different and no residues close to the upper edge of the adenine are

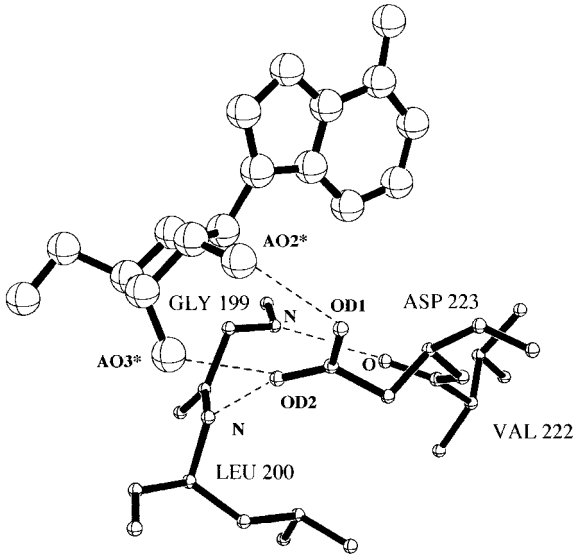
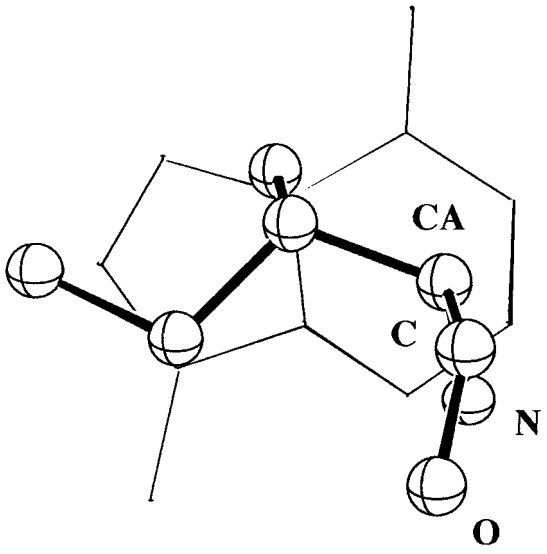


Fig. 7. View of the hydrogen bonds between the residues at site S1 and S3 and those at sites S8 and S9 (data taken from 2OHX). The first interaction between S1 and S8 forms a chair on which the NAD molecule sits; the second interaction involves the caboxilate group of the side chain of residue S9, which in turn is hydrogen bonded to the diol group of the ribose near the adenine.

found. Two anomalous situations are observed (1PSD and 2NAD), which fit neither pattern but resemble pattern 2 and show an expanded loop interacting with the B face of adenine.

Despite these different trends in adenine B face interactions, a common feature of all the structures involves a dual interaction of the S11–S14 or S15–S17 fragments with the adenine B face and the ribose at the nicotine end of NAD. No single main chain topology is observed. Indirect interactions via water molecules are also observed. The different types of interactions that occur are summarized in Figure 10.

A fourth atom cluster around the NAD involves a group of solvent atoms near the AN6 and AN1 nitrogen atoms of the adenine. They form hydrogen bonds with either of the nitrogen atoms, albeit a preference for AN1. Not all of the structures examined here contain water molecules and their presence may depend on the environment as shown in detail in Figure 11. In 1EMD a tyrosine (residue S8) precedes the aspartate S9. A water molecule (HOH 317) bridges the AN6 nitrogen atom of NAD and the OH atom of tyrosine through hydrogen bonds. In 1GEU a phenylalanine is in position S8 (Fig. 6) with a different orientation from that of the tyrosine of 1EMD. A water molecule is also present near the AN6 nitrogen atom (HOH 130) and bridges AN6 with the main chain oxygen atom of proline 231. The substitution of tyrosine with phenylalanine requires a significant local structural change.



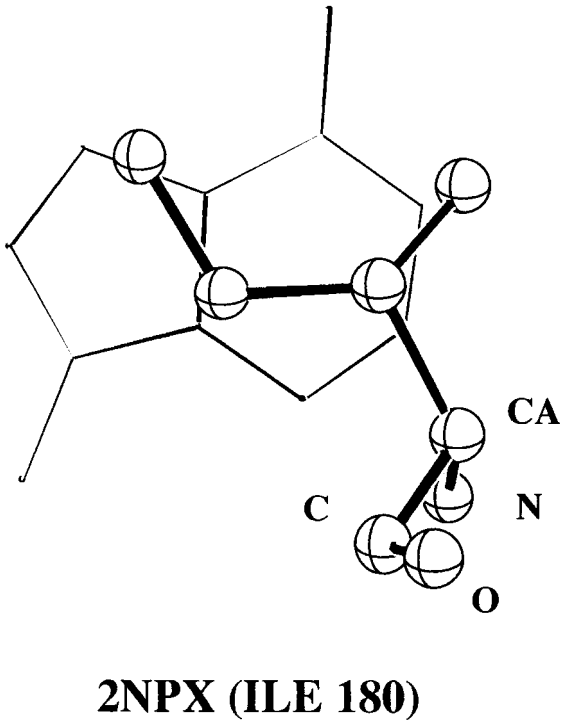


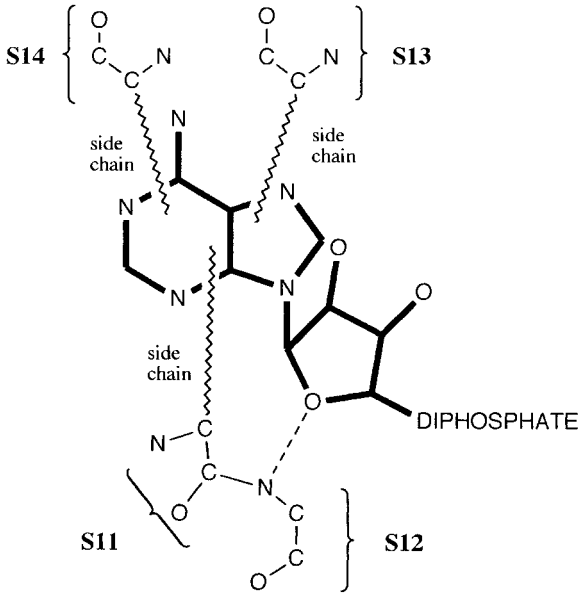
Fig. 8. Projection of the Ile side chains on the A face of the adenine of NAD for the enzymes 1BMD and 2NPX. Although the side chains have similar conformations, they approach different parts of the adenine, illustrating the nondirectionality of this protein-NAD interaction.

NADP binding pocket characteristics

Figure 12 shows the superposition of all the NADP cofactors (40 common atoms, excluding the terminal

phosphate oxygen atoms due to possible inconsistency of their labeling) and their binding pockets onto the NADP of 1PGO.

In comparison with the analogous Figure 4 for the NAD molecules, there is little evidence for atom clustering. This is in part a consequence of the great variability observed in the NADP conformations and also reflects substantial differences among the pockets surrounding NADP. Only a few general conserved trends will be subsequently discussed.



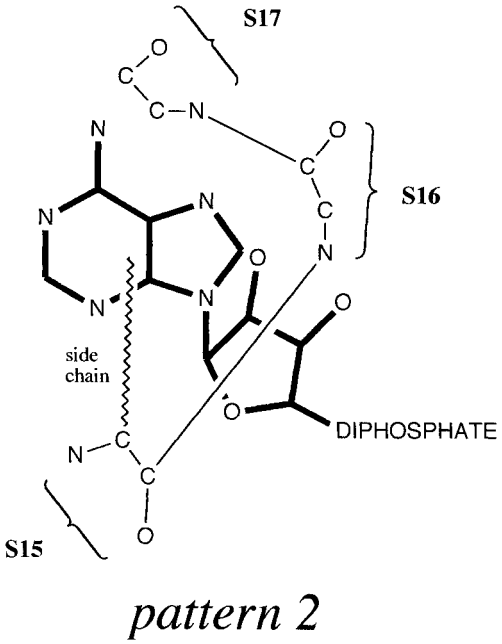


TABLE V. Sequences of the Protein Fragments Interacting With the B Face of the Adenine Moiety of the NAD Molecules

Protein data bank	Residue S11		S12		S13		S14	
Pattern 1								
1BMD	gly	87	ala	88	gln	111	ile	107
1DHR	ala	83	gly	84	thr	110	gln	106
1EMD	ala	77	gly	78	leu	101	ile	97
1HDR	ala	86	gly	87	thr	113	gln	109
1HLP	ala	96	gly	97	ile	120	ile	116
1LLD	ala	83	gly	84	ile	113	ile	107
1LDM	ala	96	gly	97	ile	120	ile	116
1LDN	ala	96	gly	97	ile	120	ile	116
2HSD*	ala	88	gly	89				
4MDH	gly	87	ser	88	gln	111	ile	107
9LDT	ala	98	gly	99	ile	123	ile	119

Protein data bank	Residue S15		S16		S17	
Pattern 2						
1GD1	thr	96	gly	97	arg	98
1GGA	thr	110	gly	111	leu	112
1HDG	thr	96	gly	97	val	98
1HDX	ile	269	gly	270	arg	271
2OHX	ile	269	gly	270	arg	271
1GEU	ile	261	gly	262		
1LVL	val	264	gly	265		
2NPX	val	242	gly	243		

*The residues in positions S13 and S14 cannot be identified with certainty.

In only 3 of 11 (1PGO, 1QOR, and 1TYP) NADP structures does a polypeptide fragment interact with the diphosphate moiety in an extended hydrogen-bonded manner similar to that observed in NAD complexes. In all the three cases the protein segment interacting with the diphosphate moiety is located in a loop between a β strand and an α helix antiparallel to it, as is also the case for NAD binding. Both geometrically and chemically, the 1PGO complex appears more similar to the NAD complexes than 1QOR which is more similar to the NAD complexes

Fig. 9. Schematic representation of the interactions between the protein and the B face of adenine of NAD. Two different patterns are generally observed. In the first, four residues at sites S11 to S14 are considered where the first two follow a β strand and the last two are in the middle of a successive antiparallel α helix. The first residue (S11) is in front of the lower part of the B face of adenine, the second (S12) forms a hydrogen bond with the endocyclic oxygen atom of the ribose near the adenine, and the third (S13) and the fourth (S14) direct their side chains toward the upper part of the B face of adenine. Only the first two residues are well conserved as either alanines or glycines. In the second pattern (bottom) three residues from S15 to S17 are considered. They follow a β strand as do the residues at sites S11 and S12 of pattern 1. The first residue (S15) directs its side chain toward the B face of adenine, whereas the other two (S16 and S17) are near the upper edge of the adenine. S17 is sometimes absent because of a different orientation of the backbone with respect to the cofactor. Only S16 is highly conserved (always a glycine) for steric reasons.

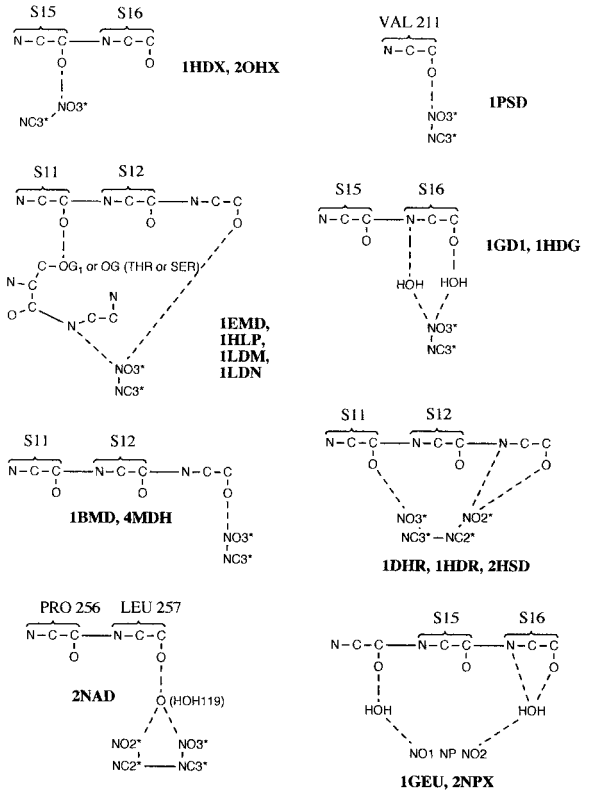


Fig. 10. Schematic representation of the interactions between the ribose near the nicotinamide moiety of NAD and the protein. The residues involved also interact with the B face of adenine, thus bridging the two ends of the cofactor. Although various types of interactions are observed, hydrogen bonds are always involved. In some cases they are indirect, utilizing a water molecule to bridge the protein and NAD.

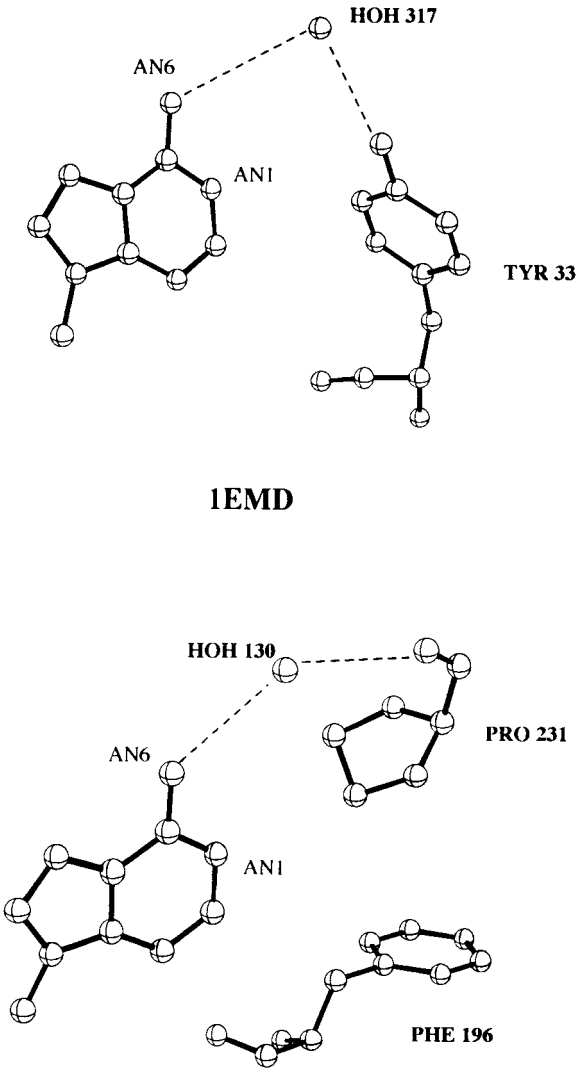


Fig. 11. Relationship between the conformation of the side chain of the residue at site S8 (Fig. 6) and the water molecule near the AN6 and AN1 nitrogen atoms of NAD illustrated with examples taken from 1EMD and 1GEU. Water molecules are often observed near the AN6 and AN1 nitrogen atoms of NAD but their exact location and their possible absence may depend on the nature of the residues surrounding them and on the conformation of the side chains of these residues.

than 1TYP, although all present a protein segment similar to the consensus sequence for the NAD diphosphate recognition. In another five cases (1DLR, 1DRH, 3DFR, 8DFR, and 1ADS) this interaction is quite different, although some general characteristics are conserved such as the secondary structure of the interacting protein fragment. In the final three NADP complexes (1CAF, 1TCS, and 1IKB) the NADP diphosphate interacts with the protein in a different way.

In 1PGO the sequence Gly 9–Leu 10–Ala 11–Val 12–Met 13–Gly 14 corresponds to the sequence reported in Table III where the residue at site S2 is missing. Gly 9 occupies S1 because its nitrogen atom is hydrogen bonded to the carbonyl oxygen of Phe 31 followed by Asn 32, which chelates the diol group of the ribose near the adenine. The main chain nitrogen of Leu 10 is hydrogen bonded to the ND2 atom of Asn 32 and therefore corresponds to the residues in positions S2 or S3. The nitrogen atoms of Val 12 and Met 13 also interact with the oxygen atoms of the NADP diphosphate moiety but indirectly through water molecules HOH 686 and HOH 692. These two residues therefore resemble those in sites S5 and S6. Moreover, Met 13, as residues in position S6, directs its side chain toward the nicotinamide fragment of NADP and is followed by Gly 14 in position S7.

In 1QOR the sequence Ala 149–Ala 150–Gly 151–Gly 152–Val 153–Gly 154 corresponds to the diphosphate binding sequences of Table 3 (again S2 missing). Many single interactions are conserved: the nitrogen atom of Gly 152 forms a bifurcated hydro-

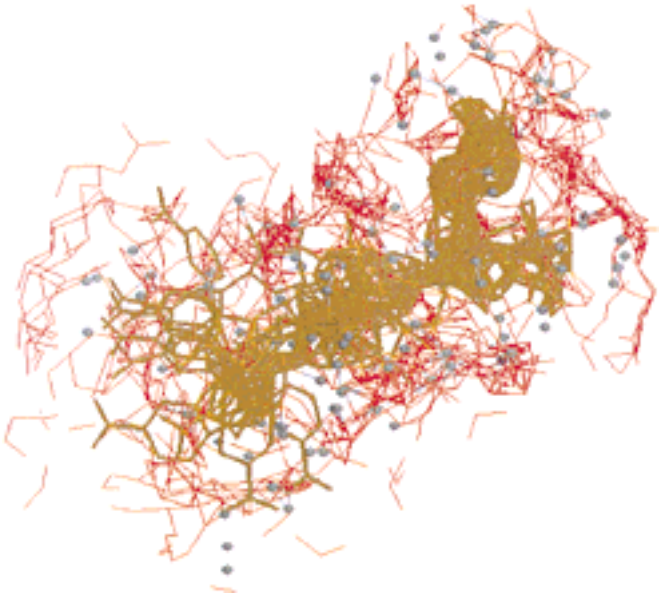


Fig. 12. Superposition of the NADP molecules (brown stick model) and of the atoms defining their pockets (red stick model = protein atoms; grey spheres = water molecules). The NAD molecules are oriented with the adenine moieties to the right and the nicotinamides to the left. No clustering of pocket atoms appears, contrary to the case for NAD (Fig. 4).

gen bond with the two diphosphate oxygen atoms, the nitrogen atom of Val 153 forms a hydrogen bond with a diphosphate oxygen atom, and the side chain of Val 153 is directed toward the nicotinamide ring. There are nevertheless differences from the protein-NAD interactions: the nitrogen atom of Ala 149 does not form a hydrogen bond with a main chain carbonyl oxygen atom of a residue distant in the sequence and the nitrogen atom of Gly 151 does not interact, directly or indirectly, with the adenine ribose. The nitrogen atom of Ala 150 is hydrogen bonded to the water molecule HOH 56, interacting with the AOP1 oxygen atom of the NADP molecule.

In 1TYP a sequence Gly 195–Gly 196–Gly 197–Tyr 198–Ile 199–Ser 200 also behaves similarly as NAD diphosphate recognition (S2 missing). Gly 196 interacts with the adenine ribose through a hydrogen bond N . . . AO3*, thus resembling the conserved glycine in position S4 (Table III). The main chain nitrogen atom of Gly 195 and the oxygen atom of Gly 196 are hydrogen bonded to the oxygen atom of Ala 220 and to the nitrogen atom of Leu 227, respectively, residues close to those that interact with the adenine ribose and A face. Both Gly 195 and Gly 196 therefore resemble the S1 residue of the NAD complexes (Table III). No bifurcated hydrogen bond bridging two diphosphate terminal oxygen atoms is formed. Only the nitrogen atom of Tyr 198 clearly forms a hydrogen bond with the NO1 oxygen atom of the NADP; Tyr 198 also directs and stacks its side chain against the nicotinamide ring and therefore acts as residues in position S6 of Table III. However, Tyr 198 is not followed by a glycine and the successive residue Ile 199 plays a relevant role in stabilizing the local packing with its side chain in front of Tyr 198.

A protein segment is also involved in binding the diphosphate moiety of the NADP cofactor in the dihydrofolate reductases (1DLR, 1DRH, 3DFR, and 8DFR), aldose reductase (1ADS), and catalase (1CAF) but the geometry and chemistry of these diphosphate-protein interactions are completely different from those observed in NAD complexes. In dihydrofolate reductases two protein segments are roughly parallel to the NADP diphosphate moiety. One of the sequences Gly-X-X-Thr (the first glycine is Gly 53 for 1DLR and 8DFR, Gly 42 for 3DFR, and Gly 43 for 1DRH) is oriented in the adenine-to-nicotine direction and the other with sequence X-Gly-Gly-X-X-X (the first glycine is Gly 116 for 1DLR and 8DFR, Gly 98 for 3DFR, and Gly 95 for 1DRH) is oriented in the opposite direction. Both protein segments interact with the NADP molecule through both main chain and side chain atoms. In 1ADS the segment Ser 214–Pro 215–Asp 216 surrounds the diphosphate of NADP forming hydrogen bonds with the terminal oxygen atoms through side chain atoms. In 1CAF the segment Trp 282–Pro 283–His 284 is nearly parallel to the diphosphate of NADP and is oriented in the nicotine-to-adenine direction and the diphosphate oxygen atoms are hydrogen bonded to both main chain and side chain atoms. A common feature of all the above (except 1CAF) is that the protein segment involved in this interaction is located in a loop

between a β strand and an α helix antiparallel to it. This suggests that the helix dipole plays a stabilizing role in the interaction with the electronegative diphosphate group.[25] However, in the other NADP-protein structures no clear evidence for such a mechanism arises. The interaction stereochemistry is also different with the diphosphate group surrounded by many single residues far from one other in the protein sequence.

The interactions between the proteins studied here and the NADP adenine are always different from those observed in the NAD complexes. This is reflected in the greater variability of the relative orientation of the adenine and adenine ribose rings. In some NADP cases the torsion around the AC1*-AN9 bond can vary by 180°, inverting the orientation of the A and B adenine faces. For example, in 1QOR where the AO4*-AC1*-AN9-AC8 dihedral angle is 130°, the B face of the adenine is directed in the same direction as the diol group of the adenine ribose, whereas in all the NAD complexes it is the A face of the adenine that is syn to the diol group of the adenine ribose. The phosphoesterification of the 2′-hydroxyl group appears responsible. The phosphomonoester characteristic of NADP hinders substantially the face of the adenine syn to it such that a side chain cannot approach the adenine of NADP and NAD with exactly the same geometry. Moreover, the phosphomonoester characteristic of NADP is negatively charged (at least partially) such that the electronic surrounding of the adenine face syn to the phosphomonoester is considerably different in NAD and NADP complexes.

The steric requirements of the diol group of the adenine ribose are also different in NAD and NADP. The phosphomonoesterification of the NADP diol influences all the interactions between the protein and the adenine ribose. In NAD complexes a carboxylate side chain chelates with the adenine ribose diol, whereas in NADP complexes only a few structures show a double interaction with both the diol oxygen atoms. In 1ADS the terminal nitrogen atom of the Lys 262 side chain is hydrogen bonded to the AO3* and AOP1 oxygen atoms and in 1PGO Asn 32 forms hydrogen bonds with both the phosphomonoester and the ribose hydroxyl group.

The successive and conserved S9 and S10 residues interacting with the adenine and adenine ribose in NAD complexes find a counterpart in NADP complexes. Very often the terminal nitrogens in the side chain of an arginine are hydrogen bonded to the terminal oxygen atoms of the phosphomonoester; the side chain is also roughly parallel to and facing the adenine plane (Fig. 13). In the dihydrofolate reductases the arginine is sometimes substituted by histidine (3DFR) or serine (1DRH). In all the dihydrofolate reductases the arginine-phosphate hydrogen bonds are indirect so that waters bridge the phosphate oxygen atom and arginine side chain nitrogen

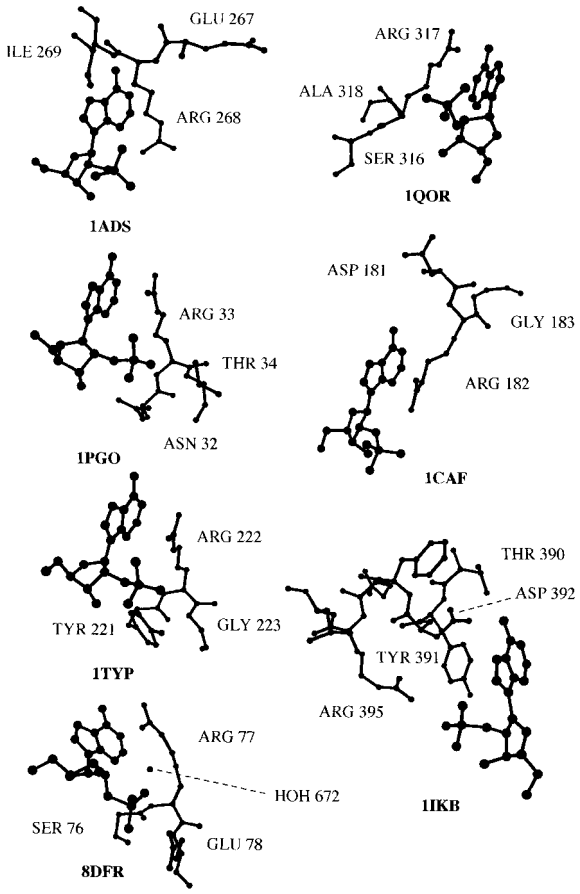


Fig. 13. Illustrations for several NADP complexes where the adenine plane generally faces an arginine side chain whose guanidinium is hydrogen bonded to the phosphomonoester of the neighbor ribose. This type of interaction displays the double character of the arginine side chain: its aliphatic chain is able to make hydrophobic interactions while the guanidinium group participates in electrostatic interactions. Few exceptions to this general rule are observed: in 1TCS no arginine is present [but 1TCS is not of particular interest because it is not a NADP-dependent enzyme] and in 1IKB the arginine side chain interacts with the phosphomonoester but does not face the adenine plane. If the interaction between the guanidinium group and the phosphomonoester is indirect, i.e., mediated by a water molecule (see 8DFR in the figure), the arginine side chain can be substituted by other residues (serine or histidine in 1DRH and 3DFR, respectively) (not shown).

atom; this probably explains the substitution of arginine by histidine or serine. Such arrangements are observed in 9 of the 11 NADP complexes. In 1IKB an arginine is hydrogen bonded to the terminal oxygen atoms of the phosphomonoester but its side chain is not stacked against the adenine. In 1TCS, where no arginine or reasonable substituents are observed near the adenine or its ribose, the NADP cofactor has no functional role. There is, however, a group of water molecules in front of the 1TCS adenine and a cluster of hydrogen bonds connects them to the phosphomonoester.

The conformations of arginine side chains and their orientation with respect to adenine are not

conserved in all the structures. The secondary structures of arginine are also quite variable. In seven of the nine structures (1PGO, 3DFR, 8DFR, 1DRH, 1DLR, 1TYP, and 1ADS) arginine is located in a loop between an antiparallel β strand and an α helix; in two cases (1CAF and 1QOR) it is at the end of an α helix, which is followed by an antiparallel β strand. Moreover, in 1QOR this arginine (Arg 317) is found at the end of a COOH-terminal domain, different from the classical dinucleotide binding fold.

The other features conserved in the NAD-protein interactions (the interaction with the B face of adenine, the bridging of the B face of adenine and the nicotine ribose, and the water molecules hydrogen bonded to the AN6 and/or AN1 nitrogen atoms of adenine) are not so well observed in the NADP complexes. Three illustrative and nonconserved examples are given in Figure 14 where in 1PGO there is no link formed between the adenine and nicotine ribose, in 1QOR there is a link similar to those found in NAD complexes, and in 1ADS there is a link that is different from the NAD types because Tyr 209 stacks against the nicotinamide.

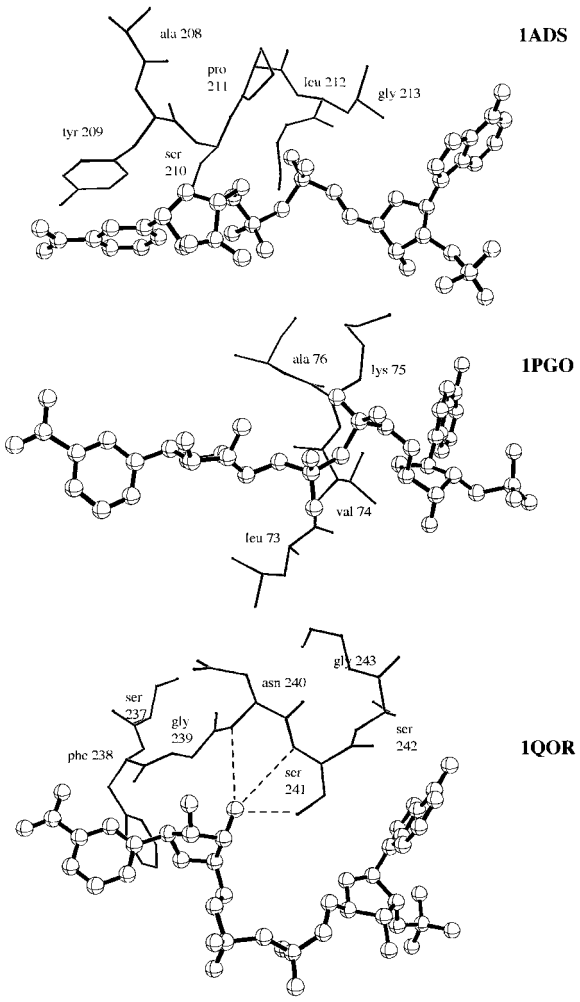


Fig. 14. Illustrations of several NADP complexes showing the residues interacting with the B face of adenine. In 1QOR and 1ADS these residues also interact with the ribose near the nicotinamide moiety, common in NAD complexes (Fig. 10); however, in 1ADS the nature of the link between adenine and ribose is atypical. In 1PGO the residues interacting with the B face of adenine do not interact with the ribose near the nicotinamide.

DISCUSSION

NAD and NADP cofactors have substantially different conformations and their interactions with proteins generally obey different rules. Moreover, the NADP complexes show a higher conformational variability than NAD and correspondingly the NADP-protein interactions seem less conserved than those for NAD-protein complexes. In both, the packing around the nicotinamide moiety is very substrate dependent. Given the hydride transfer from or to the NC4 carbon atom, both the geometry and charge distribution around the nicotinamide group would be expected to change to interact with a large variety of substrates.

NAD-protein interactions can be divided into four different categories: 1) a protein segment interacts primarily with the diphosphate moiety, 2) another protein segment ensures the fixation of the adenine ribose diol and the A face of the adenine and associates through hydrogen bonds with the polypeptide fragment cited above, 3) a group of residues in front of the B face of the adenine associates it with the nicotine ribose, and 4) water molecules tend to cluster near the AN6 and AN1 nitrogen atoms of the adenine. Among these four contributions, the first two are the most conserved.

The protein segment ensuring interaction with the diphosphate moiety has a general sequence S1-S2-S3-S4-S5-S6-S7 (Table III) where S1, S4, and S7 are normally glycines; S2 may be absent; S3 and S5 are variable; and S6 is always apolar (isoleucine 52%, valine 33%, and leucine 15%).

Among the few exceptions to the Gly-S2-S3-Gly-S5-S6-Gly pattern, 3-α,20-β-hydroxysteroid dehydrogenase (2HSD) (Table III) displays a different geometry of the NAD-protein interaction and represents the large family of short chain dehydrogenases.[26] Less extensive modifications include glycine-alanine substitutions for 1HLP and 2NAD at S1 and in 4MDH at S7. The relevance of the conserved sequence in diphosphate recognition has been previously discussed.[5,6] However, the data set examined here is much larger than those considered previously (21 NAD-protein complexes versus 8 by Baker et al.[6] and 6 by Wierenga et al.[5]). Therefore, new features have emerged.

The conservation of the apolar character of the residue S6 has been insofar unrecognized. The side chain of this residue is always directed toward the nicotinamide group of the cofactor. Moreover, Baker et al.[6] noted that the second glycine (residue S4) may

interact either directly or indirectly with the adenine ribose; in the first instance a N . . . AO3* hydrogen bond is formed, whereas in the second the glycine is hydrogen bonded to a side chain of another residue (at site S9) that is in turn hydrogen bonded to AO3*. They supposed that the indirect interaction occurs only if the last glycine (S7) is substituted by an alanine. However, the data set examined in this study suggests that the few alanine/glycine substitutions do not play a relevant role in determining the type of the diphosphate-protein interaction. There are no clear cases of direct N . . . AO3* hydrogen bonds in NAD complexes, although possible in NADP complexes, but even then only hydrogen bonds between the second glycine and an aspartate or glutamate side chain, in turn hydrogen bonded to the ribose AO3* atom, are observed.

The universal hydrogen bonding of the main chain nitrogen of the first glycine (residue S1) with the backbone carbonyl oxygen of the residue S8 preceeding the aspartate or glutamate interacting with the adenine ribose has not been previously recognized. The same glycine may also be hydrogen bonded to the AN3 nitrogen atom of adenine; the possible substitution to alanine does not modify this interaction.

The possibility of the presence or absence of residue S2 has already been noted[6] but it was considered as a peculiar exception, although we find that it is often missing (Table III). It behaves as a second residue S3 with its main chain nitrogen atom indirectly hydrogen bonded to AO3* through the aspartate or glutamate carboxylic group of residue S9. The absence of S2 does not imply substantial transformations to the diphosphate-protein interaction.

The protein segment interacting with the diphosphate moiety of both cofactors cannot be considered as a discriminator between NAD and NADP because its sequence conservation is not complete in NAD complexes (the exception of the short chain dehydrogenases) and because it is sometimes found in NADP complexes (6-phosphogluconate dehydrogenase (1PGO), quinone oxidoreductase (1QOR), and trypanothione reductase (1TYP).

Another unrecognized contribution to the NAD-protein interaction is the greatly conserved group of residues facing the A side of the adenine and the adenine ribose diol group. Three such residues are important: S8, S9, and S10 (Table IV). The first, though not conserved, interacts with the first residue (generally glycine) of the protein segment interacting with the NAD diphosphate (position S1). The second one (S9) is doubly hydrogen bonded to the adenine ribose diol and also to the S2 and S3 (if present) residues. The residue S10 interacts with the A face of the adenine. Residue S9 is well conserved (aspartate in 85% of the cases and glutamate in the remainder) obviously because of the type of hydrogen bond formed with the diol group. S10 is not very well conserved. In 85% of the cases an apolar residue is found but also methionine may be present with its thioetheric sulfur atom directed toward the adenine A face or arginine with its apolar side chain segment nearly parallel to the adenine A face. The importance of the residue S9, aspartate, or glutamate has been observed long ago[23,27] and has been considered a fingerprint for NAD binding pockets. The other observations on S8-S9-S10 are ascribable to this work.

Contrary to the NAD-protein interaction, the NADP-protein interactions cannot be logically decomposed into various conserved features, although in a few cases features common to those observed in NAD complexes appear such as the protein-diphosphate interaction in 6-phosphogluconate dehydrogenase (1PGO), quinone oxidoreductase (1QOR), and trypanothione reductase (1TYP). There is also a well conserved feature among the NADP-protein interactions; i.e., the apolar side chain portion of an arginine (sometimes histidine or serine) facing the adenine plane and hydrogen bonded to the phosphomonoester. The latter interaction may be either di rect or indirect through mediation of water molecules that bridge the guanidinium group of the arginine and the oxygen atoms of the phosphomonoester. This arginine is a likely requirement for NADP binding.

The various observations on the NAD and NADP pockets clearly show that their in vivo molecular discrimination is not a matter of changing a few residues. Early attempts at protein engineering to switch the nucleotide specificity were focused on mutations involving the acidic residue with side chain hydrogen bonded to the diol of the adenine ribose.[3,4] Other approaches[2] included subtle substitutions such as alanine for glycine at S7. Although all of these studies succeeded in shifting the coenzyme specificity, none obtained a novel enzyme as catalytically efficient as the wild type. It is clear that in any engineering or design efforts the four major clusters of atoms about the NAD pocket should be conserved for design or not disturbed in engineering. Similarly, the arginine in NADP complexes appears crucial. The pocket segment that hydrogen bonds to the diphosphate moiety in a strand-like fashion would also be an important element in designing a pocket to attract dinucleotides. Aspartate is also crucial in stabilizing the adenine ribose in NAD complexes as are hydrophobic interactions with the adenine plane.

CONCLUSIONS

The nicotinamide adenine dinucleotides NAD and NADP are ubiquitous coenzymes in biological redox reactions in all living systems and in the presence of the relevant enzyme and substrate they formally transfer a hydride ion from or to the carbon atom in position 4 of the nicotine ring in a stereospecific way. Their great structural similarity [the NADP differs from NAD only by the presence of an additional phosphate group esterified to the 2′-hydroxyl group

of the ribose at the adenine end) does not imply similar biochemistry. NAD is used almost exclusively in oxidative degradations that yield ATP and behaves therefore as an oxidant; NADP is confined, with few exceptions, to the reactions of reductive biosynthesis and behaves as a reductant. The discrimination between NAD and NADP is therefore an impressive example of the power of molecular recognition by proteins. We undertook a systematic analysis of the known crystal structures of NADP-enzyme complexes with the aim to delineate conserved or variable features in nucleotide binding as well as discriminators for NAD and NADP recognition. This class of proteins is an obvious candidate for a structural correlation study because of the large number of known complexed tertiary structures. A better understanding of the interactions between proteins and these two related cofactors would also bring interesting consequences for protein engineering and design studies, as well as provide a canonical system for understanding binding pocket development in biological systems.

The primary finding of this study is that NADP coenzymes are much more flexible than NADs in their complexes with enzymes. Moreover, although the protein-cofactor interactions are largely conserved in all the NAD complexes, they are variable in the NADP cases. In both, the pockets around the nicotinamide moiety are substrate dependent, as expected given their biochemical activity (a hydride transfer to or from one of the carbon atoms of the pyridine ring).

In the NAD complexes there are basically four regions around the cofactor in which protein-cofactor interactions are conserved. First, a protein segment within a loop between the first β strand and α helix of the canonical nucleotide binding fold ensures fixing the diphosphate group through a series of hydrogen bonds. Second, an aspartate (or less frequently a glutamate) chelates via hydrogen bonds the diol group of the ribose ring near the adenine and the successive residue, generally apolar, directs its side chain toward the plane of the adenine. The residue preceeding the aspartate and the side chain of the aspartate are hydrogen bonded to the protein segment ensuring the attachment of the diphosphate group to the protein. Third, a group of generally apolar residues in front of the B face of the adenine interacts with the diol group of the ribose ring near the nicotinamide. Fourth, water molecules are often located near the AN6 and AN1 nitrogen atoms of the adenine.

In the NADP complexes the only largely conserved structural feature is an arginine whose side chain generally stacks against the plane of the adenine and interacts with the phosphomonoester through hydrogen bonds. The elegance and simplicity of this interaction makes it a serious candidate for examination in further studies on protein-nucleotide recognition. The arginine emanates from different secondary structures over the various complexes, suggesting that it is the nature of its side chain that determines the interaction. When the arginine is occasionally substituted by other residues (histidine and serine), the interaction with the phosphomonoester is mediated by a water molecule. Only two exceptions are found: isocitrate dehydrogenases in which an arginine interacts with the phosphomonoester but is not parallel to the adenine plane and trichosantin, which is not an NADP-dependent enzyme but a NADP-protein complex.

Among the four types of interactions conserved in NAD complexes, all are seldom observed in NADP complexes. However, in some cases (6-phosphogluconate dehydrogenase, quinone oxidoreductase, and trypanothione reductase) the interaction of the diphosphate with the protein is ensured by a protein fragment within a loop between a β strand and an α helix antiparallel to it, which closely resembles the protein fragment systematically present in NAD complexes. No aspartate or glutamate residues are found to interact with the diol group of the ribose near the adenine. However, in 6-phosphogluconate dehydrogenase an asparagine does so and it is followed by an arginine that stacks against the adenine plane, similarly to the NAD complexes. There are also several NADP cases in which residues face the adenine plane and interact with the ribose near the nicotinamide or in which a water molecule is observed near the AN1 and AN6 nitrogen atoms of adenine. In only one case in NAD complexes (formate dehydrogenase) does an arginine face the adenine plane as occurs in NADP complexes.

It can be concluded that only the presence of an aspartate (or glutamate) able to chelate the diol group of the ribose near the adenine is an evident fingerprint to discriminate NAD and NADP. Moreover, the presence of an arginine able to face the adenine plane and to interact with the phosphomonoester group of NADP seems a requirement for NADP recognition, although it is not an evident mechanism for discriminating NAD and NADP.

METHODS

All the protein crystal structures present in the January 1995 version of the Brookhaven Protein Data Bank[8] were searched with the SRS query software[28,29] for NADP-bound moieties. Thirty-four NAD- and 23 NADP-containing structures were found with a total of 93 crystallographically independent subunit complexes. Atomic coordinates entries containing disordered NADP molecules or with crystallographic occupancy lower than 1.0 were disregarded; sometimes atoms missing in the electron density maps are modeled and cannot be considered experimental results. Occasionally, atomic occupancies are refined in cases of local disorder with resulting occupancies less than 1.0 and less reliable posi-

tioning than for ordered atoms. A stereochemical quality assessment of the resulting entries was achieved with PROCHECK (version 3.0).[30] Only structures with a Morris class[31] lower than 4 were retained. Entries whose percentage of residues falling in the disallowed zones of the Ramachandran plot was higher than 2.0 were also disregarded. A 2.0 cutoff was also applied to the percentage of bad contacts (with respect to the number of atoms). Entries containing structures of mutant or chemically modified enzymes were eliminated if the wild type was deposited. In cases of identical sequences, only the structure with the highest resolution was retained. When more than one monomer is present in the asymmetric unit, only the first chain was retained. Thirty-two entries were thus selected as reported in Table I; 21 one of them contained NAD moieties and the remaining 11 had NADP molecules.

The atomic labeling for the NADP molecules is the standard one used in the Protein Data Bank files (Fig. 1). The possible inconsistency of the labeling of the terminal oxygen atoms of the phosphate groups was not explicitly considered. No conventional nomenclature was used for the torsional angles within the NADP cofactors (as suggested by Eklund et al.[32] or by Arnott and Hikins[33]) because different conventions have been followed in the literature. However, a simple four-atom nomenclature of the type A/B/C/D was used such that the torsion around the bond between the B and C atoms is the angle between the normal to the plane defined by the A, B, and C atoms and the normal to the plane defined by the B, C, and D atoms (i.e., the solid angle between the two planes). The sign of the torsion angle value is positive if a clockwise rotation of the AB bond looking down BC direction is needed to bring it into overlap with the CD bond.

Superposition of three-dimensional structures was performed with the method of Kabsch[34] and McLachan.[35] Cluster analyses were performed according to Carugo[36] on the proteins listed in Table I: the proximity matrix, whose elements are the root-mean-square distance values between chemically equivalent atoms for each pair of entries, is cluster analyzed by a hierarchical agglomerative algorithm together with single linkage similarity criterion between two clusters. Thresholds are increased from 0.0 Å in increments of 0.05 Å until all the elements fall in a unique cluster; in this manner two successive cluster fusions are discriminated by a difference in the threshold value, which has physical meaning, and not only by a serial number, which is the classical clustering step and lacks an immediate interpretation. NADP binding pockets were defined by all enzyme and solvent atoms within 4.5 Å from any of the NADP atoms. Note that cluster analyses applied to all the 93 crystallographically independent subunits yielded similar results as those obtained from the data set given in Table I.

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