Crystallographic Investigations of Nicotinamide Adenine Dinucleotide Binding to Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The binding of NAD to liver alcohol dehydrogenase has been studied in four different ternary complexes by using crystallographic methods. These complexes crystallize isomorphously in a triclinic crystal form which contains the whole dimer of the enzyme in the asymmetric unit. This form of the enzyme has been refined at 2.9-Å resolution to a crystallographic *R* factor of 0.22. NAD binds in essentially the same way in these complexes. The binding site is located at the central part of the coenzyme binding domain. The adenine ring binds with hydrophobic interactions between two isoleucine side chains. Both ribose rings have ^2E(C2′-endo) puckering, and each ribose makes three hydrogen bonds to the enzyme. The pyrophosphate bridge has hydrogen bonds to the side chains of arginine-47 and -369 and to main chain nitrogen atoms from the amino ends of two α-helices. The nicotinamide ring is in van der Waals contact with the active-site zinc atom and with the sulfur atoms of its cysteine ligands. The carboxamide group is about 30° out of the plane of the nicotinamide ring and hydrogen bonds to main chain atoms of residues 292, 317, and 319. The overall conformation of the NAD molecule is similar to that observed for other dehydrogenases, but differs in details. In the presence of the coenzyme, the enzyme undergoes a large conformational change from an open to a closed form. This conformational change has three major effects: to create favorable binding interactions with groups of the enzyme, to enclose the coenzyme and gain binding energy for the coenzyme by reducing the accessible surface area, and to close off one entrance to the active site. As a comparison, ADP-ribose binding has been studied in the open form of the enzyme. The adenosine moiety binds in a similar way as NAD, while the rest of the molecule has different interactions.

Coenzyme binding to NAD-dependent dehydrogenases has been studied extensively during the last decade by a variety of methods [for recent reviews, see Everse et al. (1982) and Brändén & Eklund (1980)]. The preliminary X-ray structures of lactate, malate, alcohol, and glyceraldehyde-3-phosphate dehydrogenases provided important new information on the general nature of NAD–enzyme interactions [for a review, see Rossmann et al., (1975)]. It was found that the NAD binding domains of these enzymes have a similar fold with certain important invariant residues. This common structure binds NAD in a similar way and with a similar extended conformation (Ohlsson et al., 1974; Rossmann et al., 1974, 1975).

In these comparisons, the binding of the coenzyme to alcohol dehydrogenase was not known but was deduced from the binding of ADP-ribose (Nordström & Brändén, 1975; Abdallah et al., 1975). Complexes with the full coenzyme gave crystals of a different space group. The structure of the enzyme in this space group was later solved. The protein undergoes a large conformational change where the catalytic domain makes a large rigid-body rotation toward the coenzyme binding domain and a loop in the coenzyme binding domain changes drastically (Eklund et al., 1981).

In order to obtain a detailed description of coenzyme binding to alcohol dehydrogenase, we have carried out a crystallographic refinement of a triclinic crystal form of a complex with NADH and the inhibitor dimethyl sulfoxide (Me_2SO).[1] The results of this refinement allow us to describe the detailed binding of the coenzyme in this complex as well as in isomorphous complexes with NAD^+–bromobenzyl alcohol, NAD^+–pyrazole, and NAD^+–4-iodopyrazole, for which the binding of substrate and inhibitors has been described recently (Eklund et al., 1982a,b). The present crystallographic *R* factor is in the best case 0.22.

Since coenzyme binding plays a key role in the conformational transition of the enzyme, we also describe the empty coenzyme binding site in the apoenzyme and the binding of a coenzyme fragment in the isomorphous ADP-ribose complex in which the conformational change has not taken place.

Experimental Procedures

The ternary complexes described in this paper (Table I) form triclinic crystals with cell dimensions a = 52.0 Å, b = 44.5 Å, c = 94.3 Å, α = 104.5°, β = 102.0°, and γ = 70.8°. These crystals contain one dimer per asymmetric unit, and thus the structures of two subunits of the molecule are independently determined. A total of 16 344 reflections corresponding to a resolution of 2.9 Å were measured for every ternary complex on a Stoe four-circle diffractometer by using procedures described earlier (Eklund et al., 1976).

The structure of the triclinic ternary complex of alcohol dehydrogenase, NADH, and Me_2SO was originally solved by multiple isomorphous replacement (Eklund et al., 1981). The coenzyme in this complex was fitted to the electron density map in a Vector General 3404 interactive graphics display using FRODO (Jones, 1978). The structure of this ternary complex was subsequently refined with five cycles of constrained–restrained least-squares minimization using the program CORELS (Sussmann et al., 1977) to give an *R* factor of 0.26. This refinement was done without including water molecules and using an overall temperature factor.

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[1] Abbreviations: LADH, liver alcohol dehydrogenase; LDH, lactic acid dehydrogenase; *S*-lac-LDH, lactic acid dehydrogenase complex with (3*S*)-5-(3-carboxy-3-hydroxypropyl)nicotinamide adenine dinucleotide (oxidized); GPDH, glyceraldehyde-3-phosphate dehydrogenase; F_o, observed structure factors; F_c, calculated structure factors; rms, root mean square; Me_2SO, dimethyl sulfoxide; NMN, nicotinamide mononucleotide; s-MDH, soluble malate dehydrogenase.

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Table I

	space group	content/ asymmetric unit (daltons)	resolution (Å)	ref for further exptl details
apoenzyme	orthorhombic $C222_1$	40 000	2.4	Eklund et al. (1976)
LADH–ADP-ribose	orthorhombic $C222_1$	40 000	2.9	Abdallah et al. (1975)
LADH–NADH–Me_2SO	triclinic $P1$	81 000	2.9	Eklund et al. (1981)
LADH–NAD^+–pyrazole	triclinic $P1$	81 000	2.9	Eklund et al. (1982b)
LADH–NAD^+–4-iodopyrazole	triclinic $P1$	81 000	2.9	Eklund et al. (1982b)
LADH–NAD^+–bromobenzyl alcohol	triclinic $P1$	81 000	2.9	Eklund et al. (1982a) Plapp et al. (1978)

After every second cycle of the refinement, the structure was refitted on the display, and the coenzyme molecules were repositioned. Maps were calculated by using model phases and both $|F_o| - |F_c|$ and $2|F_o| - |F_c|$ amplitudes. These maps were used in parallel during model building. The structure factors and the corresponding phases were calculated with and without any contribution from the atoms of the coenzyme molecules. After changes during model building, the coenzyme was regularized by using FRODO. The dictionary for the coenzyme in this program was written by using standard parameters (Reddy et al., 1981; Arnott & Hukins, 1972) and a naming convention based on the new IUPAC–IUB nomenclature for polynucleotides (IUPAC–IUB Joint Commission on Biochemical Nomenclature, 1983). We included ^2E(C2′-endo) and ^3E(C3′-endo) as different possible puckerings for the ribose rings as special entries in the FRODO dictionary. This allows simple changes from one pucker conformation to another by renaming the coenzyme type (Jones, 1982). No other ribose puckering was considered.

In early stages, the different rings and phosphate groups were refined as rigid groups by using CORELS. Bond distances and angles of the coenzyme molecule were regularized after the refinement.

The conformation of the coenzyme in the refined triclinic LADH–NADH–Me_2SO complex was used as the starting model for model building of the other triclinic complexes used in this investigation (Table I) as well as for the LADH–H_2NADH–*trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde complex (Cedergren-Zeppezauer et al., 1982) and the NADH complex with active-site metal-depleted enzyme (Schneider et al., 1983).

The coenzyme molecules were rebuilt, if necessary, from $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ maps without contribution of coenzyme atoms to the F_c and phase calculation. The different parts of the coenzyme (adenine, riboses, phosphates, and nicotinamide ring) were positioned by using the interactive real-space refitting option in FRODO (Jones & Liljas, 1984). The coenzyme molecules were then regularized before the final detailed modeling.

Using CORELS results in the buildup of errors in both bond lengths and bond angles between adjacent residues in the chain. Therefore, as a last step, 45 refinement cycles with the EREF program (Jack & Levitt, 1978) were run on the NAD^+/NADH–bromobenzyl alcohol complex. This reduced the crystallographic R factor to 0.22 and improved the geometry of the model. The coenzyme molecules were included in this refinement.

Assignment of water molecules to the small densities not occupied by enzyme or coenzyme atoms is uncertain at 2.9-Å resolution. We have only included a few water molecules from the final density maps in the regions described in this paper. In these cases, hydrogen bonding and high density level support the interpretation.

Although the two subunits are chemically identical, they are crystallographically independent in the triclinic crystals and have been treated independently during refinement. There are some structural differences between the two subunits, but they are small. We have therefore, in most cases, chosen to describe the average situation and to give average values in corresponding tables.

The orthorhombic apoenzyme was refined by a number of techniques (Diamond, 1971; Deisenhofer & Steigeman, 1975; Jack & Levitt, 1978; Sussmann et al., 1977; Hendrickson & Konnert, 1980). The present R factor is 0.20 at 2.4-Å resolution. Results of the refinement will be published elsewhere.

We also reexamined the binding of ADP-ribose to this orthorhombic crystal form. The X-ray diffraction data for the complex were collected to 2.9 Å (Nordström & Brändén, 1975; Abdallah et al., 1975). In the present study, the amplitudes of the orthorhombic LADH–ADP-ribose complex were combined with phases calculated from the present refined native orthorhombic model. $|F_o| - |F_c|$ and $2|F_o| - F_c|$ maps were examined by using computer graphics. The ADP-ribose molecule was fitted into these maps, and the side chains of the enzyme residues were repositioned when necessary. The resulting model, including the ADP-ribose molecule, was refined by using the DERIV program (Jack & Levitt, 1978) with subsequent regularization (Jones, 1978; Hermans & McQueen, 1974). This reduced the R factor at 2.9-Å resolution to 0.20.

To compare the different structures, we transformed the coordinates of the triclinic complexes to the orthrorhombic LADH–ADP-ribose complex by overlapping the C_α atoms in both the coenzyme binding domains according to a least-squares program written by M. G. Rossman (Rao & Rossmann, 1973). The resulting coordinate sets were vectorized by using a program linked to FRODO to allow the simultaneous inspection of several coordinate sets (Jones, 1982). Stereodiagrams were drawn with a Hewlett-Packard plotter connected to the display system using programs written by T. A. Jones. van der Waals surfaces were produced with FRODO by using an algorithm written by B. Bush (Merck, Rahway, NJ). Accessibility calculations were made with a program supplied by F. M. Richards and T. Richmond (Lee & Richards, 1971).

Coordinates of the apoenzyme, the ADP–ribose complex, and the LADH–NADH–Me_2SO complex have been deposited at the Brookhaven data bank.

Results and Discussion

The difference electron density maps of the coenzyme region in the ternary complexes are easy to interpret (Figure 1a,b). All side chains around the coenzyme are clearly visible. All parts of the ADP-ribose molecule are easy to place in the Fourier maps (Figure 1c). Some side chains of the protein around the coenzyme binding site, such as Arg-47 and Ile-224, are flexible in the apoenzyme and have weak density. These have a fixed orientation and are clearly visible in the ADP-ribose complex.

Conformation of the Coenzyme When Bound to Alcohol Dehydrogenase. The nomenclature for the coenzyme atoms and torsion angles, which are adopted from the new IUPAC–

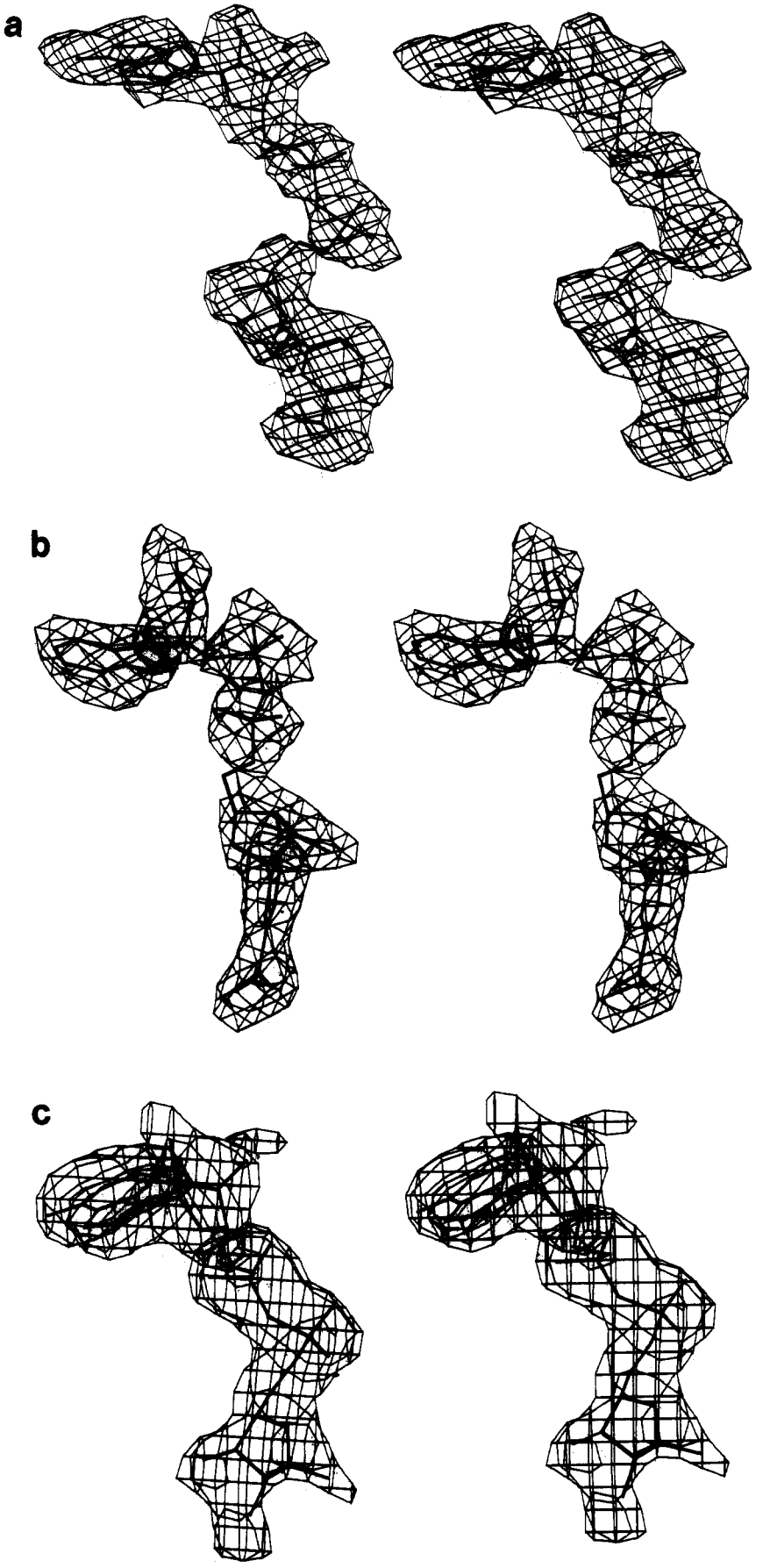

FIGURE 1: (a and b) NADH molecules in subunits 1 and 2 in the LADH–NADH–Me_2SO complex fitted into $|F_o| - |F_c|$ electron density maps. The maps were computed without contribution from coenzyme atoms and contoured at the 3σ level. The view in (b) is chosen so the deviation from planarity between the nicotinamide ring and the carboxamide is visible. (c) An ADP-ribose molecule fitted into a $2|F_o| - |F_c|$ electron density map calculated without contribution from atoms in the free ribose of the ADP-ribose molecule. the extended electron density on the O2′ atom of the free ribose is part of the electron density for a water molecule between this oxygen atom and the side chain of His-51.

Table II: Conformational Parameters of NADH, NAD^+, and ADP-ribose (ADPR) Bound to Alcohol Dehydrogenase (LADH), NAD Bound to Lactate Dehydrogenase (LDH), Soluble Malate Dehydrogenase (s-MDH), and Glyceraldehyde-3-phosphate Dehydrogenase (GPDH), NADPH Bound to Dihydrofolate Reductase (DHFR), FAD Bound to Glutathione Reductase (GR) and *p*-Hydroxybenzoate Hydroxylase (*p*-HBH), and the Small-Molecule Structure of Li-NAD^+

	χ_a	χ_a[b]	γ_a	β_a	α_a	ζ_a	ζ_n	α_n	β_n	γ_n	χ_n	χ_n[b]	pucker a	pucker n	ref	resolution (Å)	refinement
LADH + NAD1	−96	147	−79	147	106	85	−153	59	−146	39	−102	142	^2E	^2E		2.9	refined R = 0.22
LADH + NAD2	−114	135	−99	136	104	83	−151	76	−169	42	−104	139	^2E	^2E		2.9	refined R = 0.22
LADH + ADPR	−105	136	−90	124	131	45	−102	−64	179	174			^2E	^2E		2.9	refined R = 0.20
S-lac-LDH		109	124	177	−94	176	117	34	−164	31		159	^3E	^2E	Grau et al. (1981)	2.7	
LDH		89	−78	−159	109	−156	49	87	−171	−53		106	^3E	^3E	Chandrasekhar et al. (1973)	3.0	
s-MDH 1		110	−78	−167	58	−166	88	147	160	−40		132	^3E[a]	^2E	Webb et al. (1973)	2.5	
s-MDH 2		115	−74	−150	67	−156	84	160	150	−61		133	^3E[a]	^2E	Webb et al. (1973)	2.5	
GPDH 1		−177	−104	140	131	162	118	12	107	107		−77	^2E	^3E	Moras et al. (1975)	2.9	
GPDH 2		−25	−166	122	135	152	135	17	129	110		−69	^3E	^2E	Moras et al. (1975)	2.9	
DHFR		105	−169	−163	−72	141	82	57	129	−172		117	^3E	^2E	Filman et al. (1982)	1.7	refined R = 0.15
GR		132	−40	−123	−15	−160	18	−111	150				^2E		Schulz et al. (1982)	2.0	
p-HBH		121	8	−154	−22	−148	−51	140	−179				E		Wirenga et al. (1983)	2.5	refined R = 0.34
Li-NAD^+	−121		48	163	−125	133	72	79	179	47	−165		^2E	^3E	Reddy et al. (1981)	1.09	refined R = 0.10

[a] The refined structure has ^2E puckering (Birktoft et al., 1982). [b] Angles defined by Arnott & Hukins (1962).

IUB nomenclature for polynucleotides (IUPAC–IUB Joint Commission on Biochemical Nomenclature, 1983), is shown in Figure 2. The conformation of the coenzyme when bound to alcohol dehydrogenase is in general terms open, not closed as frequently found in solution. In this respect, it is very similar to other dehydrogenases (Rossmann et al., 1975) but is less extended than the NADPH bound to dihydrofolate reductase (Matthews et al., 1979). The distance between C6 of the adenine ring and the C2 atom of the nicotinamide ring is a frequently used measure of the extension of the coenzyme molecule (Rossmann et al., 1975). In LADH, we have a value of 15.0 Å, compared to 17.1 Å for NADPH in dihydrofolate reductase (Matthews et al., 1979).

Both the adenine ring and the nicotinamide ring are in anti conformations, and their planes are roughly perpendicular to the corresponding ribosome planes. The coenzyme is shaped like a boomerang, and the plane of the adenine ring is almost perpendicular to the plane of the nicotinamide ring. Thus, the planes of the two ribose rings are also roughly perpendicular to each other (Figure 1).

Although the NAD molecule is a pseudosymmetrical molecule, the conformation deviates from this symmetry when bound to alcohol dehydrogenase. The two nucleoside parts are on the same side of the phosphates (a hypothetical torsion angle defined by O5′–P–P–O5′ is in the syn range) instead of on the opposite side (anti) as in Li-NAD (Reddy et al., 1981) and NADPH in dihydrofolate reductase (Filman et al., 1982). While the AMP half is rather extended, the NMN half is bent in such a way that the ribose is enclosed more tightly between the phosphate and the nicotinamide ring. The distance between the phosphorus atom and the N1 atom of the nicotinamide ring in the NMN part is 1.3 Å shorter than the corresponding distance on the AMP half. The bending of the NMN half gives rise to close contacts mainly between O5′N, C2′N, and C3′N of the ribose and C6N of the nicotinamide ring.

The phosphate groups in the diphosphate bridge are staggered in such a way that the OP2A oxygen atom is between and in van der Waals contact to oxygens OP1N and OP2N. Since the ribose moieties are located on the same side of the diphosphate bridge, both C5′ and O5′ atoms of each ribose are close to each other.

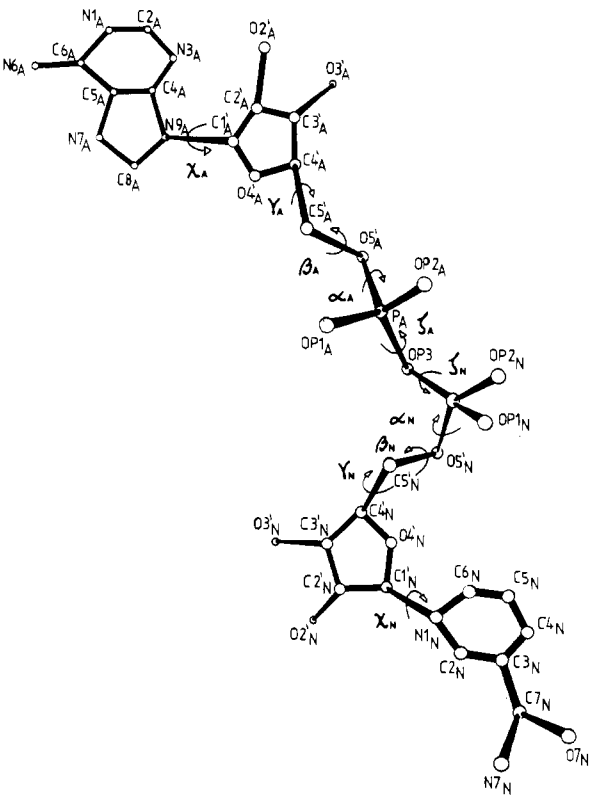

FIGURE 2: Schematic drawing of an NAD molecule with the names of coenzyme atoms and torsion angles used in this paper. The names are adopted as close as possible to the new IUPAC–IUB nomenclature for polynucleotide chains (IUPAC–IUB Joint Commission on Biochemical Nomenclature, 1983).

The torsion angles obtained for the coenzyme in each subunit and the ADP-ribose molecule are given in Table II. Angular values are sensitive to small deviations in atomic parameters, and the limited resolution of this study is a drawback. However, we have two independently refined co-

enzyme molecules in four different complexes, and the deviations are generally small. The mean deviation from the average value of the torsion angles is 8° for the eight coenzymes in this investigation. The largest difference is for the two independent subunits regardless of the complex.

As seen from the conformational parameters described in the literature (Table II), the conformation of NAD and related coenzymes varies considerably. Some of this variation is probably due to errors in the models since only dihydrofolate reductase has been crystallographically refined. The χ_a angle for alcohol dehydrogenase is similar to χ_a for FAD bound to glutathione reductase (Schulz et al., 1982). α, β, and γ for the AMP half are roughly similar to NAD bound to other dehydrogenases, such as LDH and GPDH, while these angles for the NMN part are very similar to those described for *S*-lac-LDH (Grau et al., 1981). It is obvious that the coenzyme molecule, although it has energetically preferred regions of torsion angles, can adopt to the geometry of the binding sites in the different enzymes.

The present investigation shows that both ribose sugar rings have ^2E(C2′-endo) puckering in the ternary complexes. This puckering fits the density best for both riboses (Figure 1). The assignment of puckering is very clear for the adenosine ribose for steric reasons (see below). The pucker of the NMN-ribose was uncertain in the isomorphous map, but refinement has shown that the ^2E conformation has a better fit to the electron density. This ribose binds in a narrow site, where a ribose in the ^2E conformation also makes more favorable interactions between coenzyme and protein. ^2E puckering is at least as frequently reported as ^3E conformation for coenzymes bound to enzymes (Table II).

The conformation of ADP-ribose when bound to alcohol dehydrogenase is very similar to the AMP part of the full coenzyme while the rest differs considerably and has a conformation different from that of coenzymes. ADP-ribose has the same ribose puckering as the full coenzyme. The adenosine binding site for ADP-ribose is the same as for NADH, and consequently, the only possible pucker for this ribose is ^2E. The second ribose does not have similar constraints in this binding site, which is open in this conformation of the enzyme. The ^2E conformation best fits the electron density. The puckering of both riboses was originally described as compatible with ^3E (Nordström & Brändén, 1975; Abdallah et al., 1975).

A new method was recently described for determining the conformation of NAD^+ bound to liver and yeast alcohol dehydrogenase in solution (Gronenborn & Clore, 1982). By use of proton–proton-transferred nuclear Overhauser enhancement, the conformations around the adenosine glycosidic bond and the nicotinamide glycosidic bond were determined to be in the anti conformation in both cases. The estimated torsion angles of χ_a and χ_n of about −120° are close to the values observed in our crystallographic study. However, they conclude that both riboses have ^3E conformation.

The electron density suggests that the nicotinamide ring is planar. This does not exclude the possibility that deviations from planarity (Oppenheimer et al., 1978) occur at intermediate stages during catalysis (Cook et al., 1981). Nitrogen and oxygen atoms in the carboxamide group are assigned from the formed hydrogen bonds (see below). The C7N–N7N bond of the carboxamide is thus roughly transoriented with respect to the C3N–C4N bond, which is also the conformation for NAD(P) bound to LDH (Grau et al., 1981), s-MDH (Webb et al., 1973), and dihydrofolate reductase (Filman et al., 1982). The carboxamide group is roughly coplanar to the nicotinamide ring as in NADPH bound to dihydrofolate reductase (Filman et al., 1982) and in small molecule structures (Karle, 1961; Koyama, 1963). However, the best fit to the electron density is obtained when the carboxamide is turned out of the plane of the ring by about 30° around the C3N–C7N bond. The torsion angle defined by C2N–C3N–C7N–O7N then has a value of −150°. This also improves the angles of the hydrogen bonds from the carboxamide, especially the hydrogen bonds from the nitrogen atom to the main chain oxygens which become roughly planar with the carboxamide group. This interpretation is supported by theoretical energy calculations for NADH, which have minima at 0° and ±150° for this angle (Perahia et al., 1974), and by X-ray structure determination of model small molecule structures (Wright & King, 1954; Freeman & Bugg, 1974).

After least-squares alignment of the C_α atoms of the two independent subunits, the rms deviation is 0.5 Å. With this alignment, the adenine, AMP-ribose, phosphates, NMN-ribose, and nicotinamide moieties of the coenzyme show rms differences of 0.67, 0.70, 0.68, 0.47, and 0.29 Å, respectively. It is not surprising that the deviations are larger in the AMP part, which is close to the surface of the enzyme, since catalysis requires a proper positioning of the nicotinamide ring but not of the adenine ring.

Binding Sites. The coenzyme molecule binds in the large cleft region between the two domains of the alcohol dehydrogenase subunit (Eklund et al., 1981; see also Figure 10) and interacts with residues from both domains. Most of the coenzyme binds between the two domains of the subunit while the adenosine moiety of the coenzyme binds in a cleft in the coenzyme binding domain. The clefts are approximately 30 Å long and 7 Å in diameter and extend in both directions from the coenzyme binding site. At the nicotinamide site, the cleft has a natural continuation into the substrate binding site. The extension of the cleft at the adenine binding site (Figure 3) is probably a consequence of the structural architecture of the coenzyme binding domain, and there is no obvious function for this 5-Å-long extension. The adenosine binding site is easily accessible from solution while the nicotinamide binding site is in the center of the molecule, buried far in the protein.

The coenzyme molecule binds with its pyrophosphate part at the center of the carboxyl end of the parallel pleated sheet of the coenzyme binding domain. This site is created by the two $(\alpha\beta)_3$ units of the coenzyme binding domain (Figure 4) and is commonly found for many enzymes with parallel pleated sheet structures (Brändén, 1980). The binding site for the nicotinamide ring is adjacent to the first two helices of the domain, αA and αB, with the nicotinamide ring in contact with Thr-178 from αA and Val-203 from αB. The binding site for the adenine ring is along the αD helix. The loops at the end of the βB, βC, βE, and βF strands form other sides of the binding sites for these rings. The loops between the secondary structure elements are important for the binding of the coenzyme and contain both side chains involved in adenine binding, conserved glycine residues and main chain atoms that hydrogen bond to the coenzyme. Furthermore, the loop after βE is important for the conformational change.

This general description of the binding site is, to a large extent, relevant to other known dehydrogenases but differs considerably in details (Ohlsson et al., 1974; Rossmann et al., 1974, 1975; Brändén & Eklund, 1980; Eklund et al., 1981).

(*A*) *Adenine Binding.* The adenine ring is sandwiched, in van der Waals contact, between two isoleucine side chains of residues 224 and 269 which line the cleft on both sides (Figure 5). In the interior of the cleft, the adenine ring makes van der Waals contacts with main chain atoms at the carboxyl end

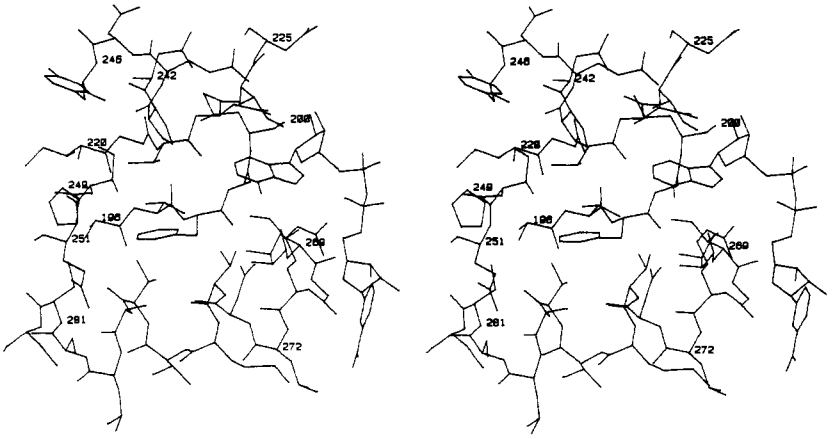

FIGURE 3: Stereodiagram showing the extension to the left of the adenine binding site in the coenzyme binding domain.

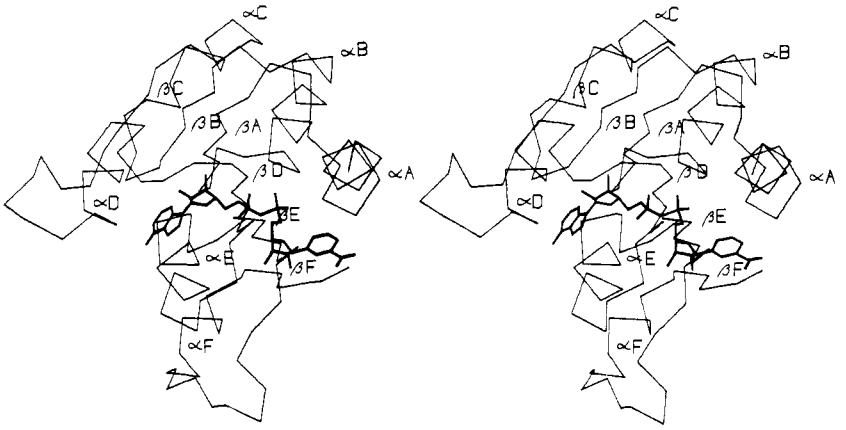

FIGURE 4: Coenzyme binds at the carboxyl end of the coenzyme binding domain. In the stereodiagram, the C_α atoms of the coenzyme binding domain are connected with thin lines and the coenzyme atoms with thick lines. The nomenclature is adopted to the regular character of the domain in alcohol dehydrogenase and is slightly different from the one used for LDH and in comparisons (Rossmann et al., 1975). The secondary structure elements, which are assigned on the basis of main chain dihedral angles, contain the following residues: βA, 193–198; βB, 218–223; βC, 239–242; βD, 264–267; βE, 287–291; βF, 312–318; αA, 174–187; αB, 202–214; αC, 226–235; αD, 250–258; αE, 272–280; αF, 305–310.

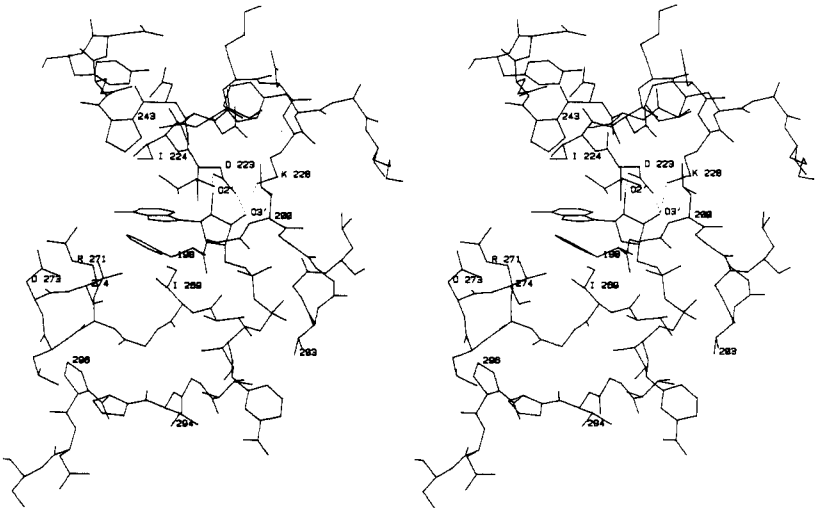

FIGURE 5: Stereodiagram viewed approximately perpendicular to the view in Figure 3 showing the adenosine binding site of the coenzyme binding domain with the bound NADH molecule. The adenine ring fits into the slit between Ile-224 and Ile-269. Arg-271 covers part of the surface of the ring from the surrounding solvent. The adenosine binding site has Asp-223 in a central position. Hydrogen bonds are drawn as dotted lines.

of βB (Table III). Asp-223, which is very important for adenosine ribose binding, also makes van der Waals contacts to atoms of the adenine ring. Atom N3A fits into the space betwen CA and OD2 of Asp-223. N6A of the adenine ring points toward the solution and is in van der Waals contact with the side chain of Arg-271. The arginine which forms an ion pair with Asp-273 covers part of the surface of the adenine edge which is directed toward the solution. The combination of a mainly nonpolar adenine binding site with the positive charge of Arg-271 has been suggested as the reason for the perturbation of the adenine spectra in ADP-ribose and coenzyme (Subramanian et al., 1981).

Table III: Contacts between Atoms of the Enzyme and Atoms That Are Closer than 3.8 Å in Any of the NAD Molecules and the ADP-ribose Molecule

	NAD	ADP-ribose
	(A) Adenine Ring	
N1A	Ile-224 CG2	Ile-224 CG2
	water	W343
C2A	Val-222 O	
	Asp-223 CA	
	Ile-224 N, CG1	Ile-224 CG2
N3A	Gly-199 N, CA	
	Val-222 O	
	Asp-223 C, CA, OD2	Asp-223 CA, OD2
	Ile-224 N, CG1	
	Ile-269 CD1	Ile-269 CD1
C4A	Ile-224 CG1	
	Ile-269 CD1, CG1	Ile-269 CD1
C5A	Ile-224 CD1, CG1	
	Ile-269 CD1	
C6A	Ile-224 CD1, CG1, CG2	
N6A	Arg-271 NH1	Arg-271 NH1
N7A	Ile-269 CG2	
	water	W347
C8A	Ile-269 CG2	Ile-269 CG2
	water	W347
N9A		Ile-269 CG2
	(B) Adenosine Ribose	
C1′A	Asp-223 CG, OD1, OD2	Asp-223 CG, OD1, OD2
C2′A	Asp-223 OD1, OD2	Asp-223 OD2
O2′A	Asp-223 CG, OD1, OD2	Asp-223 CG, OD2
	Lys-228 NZ	
C3′A	Asp-223 OD1	Asp-223 OD1, OD2
	Lys-228 NZ	
		W350
O3′A	Leu-200 N	Leu-200 N
	Gly-201 N, CA	
	Asp-223 CG, OD1, OD2	Asp-223 CG, OD1, OD2
	Lys-228 CD, CE, NZ	Lys-228 NZ
C4′A		
O4′A	Gly-199 CA	Gly-199 CA
	Asp-223 OD1	
	Ile-269 CG1	Ile-269 CG1, CG2
C5′A		
O5′A		
	(C) Pyrophosphates	
PA		W48
OP1A	Arg-47 CG, CD, CZ, NH1	Arg-47 CZ, NH2
		W48
		W55
		W355
OP2A	Gly-201 CA	Gly-201 CA
	Gly-202 N	
	Leu-362 CD1	
		W350
OP3	Arg-47 CB	
PN		
OP1N	Cys-46 CB	
	Arg-47 N	
	Arg-369 NH1, NH2	
		W10
OP2N	Gly-201 C	Gly-201 CA, C
	Gly-202 N, C, CA	Gly-202 N
	Val-203 N, CG2	Val-203 N
	Arg-369 NH1	

	NAD	ADP-ribose
	(D) NMN-ribose	
C1′N	Gly-293 CA	Gly-293 CA
	Val-294 N	
		W52
		W351
O1′N		292 O
		293 CA
		W52
		W351
C2′N		W351
		W352
		W354
O2′N	Ser-48 N, CA, CB, OG	
	His-51 CD2, NE2	
	Val-294 CB	
		W352
		W354
C3′N	Arg-47 CB	
	Ile-269 O	Ile-269 O
O3′N	His-51 CE1, NE2	
	Ile-269 C, O	Ile-269 C, O
		Gly-270 CA
	Val-294 N	
C4′N		Val-268 O
	Ile-269 O	Ile-269 C, O
O4′N		Val-268 C, O
		Ile-269 O
	Gly-293 CA	
C5′N	Val-203 CG2	
	Val-268 O, CG2	
O5′N	Val-203 CG2	Val-268 O
	(E) Nicotinamide	
N1N		
C2N	Val-292 O	
	Val-294 CG2	
C3N	Val-292 O	
C4N	Cys-174 SG	
	Thr-178 OG1	
C5N	Cys-46 SG	
	Thr-178 OG1	
	Val-203 CG1, CG2	
	Zn (active site)	
C6N	Cys-46 SG	
	Val-203 CG1, CG2	
C7N	Val-292 O	
	Ala-317 O	
	Ile-318 CA	
	Phe-319 N	
O7N	Ile-318 C, CA, CB, CG1	
	Phe-319 N	
N7N	Val-292 C, O	
	Gly-317 C, O	
	Ile-318 CA	

Only four atoms of the adenine ring have parts of their surfaces accessible to solvent (Table IV). The final electron density map shows peaks which can be interpreted as water molecules forming hydrogen bonds to N1A, N6A, and N7A, respectively. No hydrogen bond is formed between the adenine ring and the protein.

The adenine binding site in alcohol dehydrogenase is not specific for adenine and can accommodate many other hydrophobic groups (Einarsson et al., 1974; Biellmann et al., 1979; J.-P. Samama and H. Eklund, unpublished results). This is in contrast to other enzymes, for example, citrate synthase, which has a specific hydrogen bond pattern for adenine (Remington et al., 1982).

(*B*) *Adenosine Ribose Binding.* The adenosine ribose binding site is at the surface of the coenzyme binding domain. Glycine residues at positions 199, 201, and 202 allow the coenzyme to come close to the main chain. The first two of these residues are conserved in all alcohol dehydrogenases (Jörnvall et al., 1978; Dennis et al., 1984) and in other dehydrogenases (Rossmann et al., 1975). In a mutant of alcohol dehydrogenase from *Drosophila*, the position corresponding to position 199 is substituted by an Asp (Thatcher, 1980),

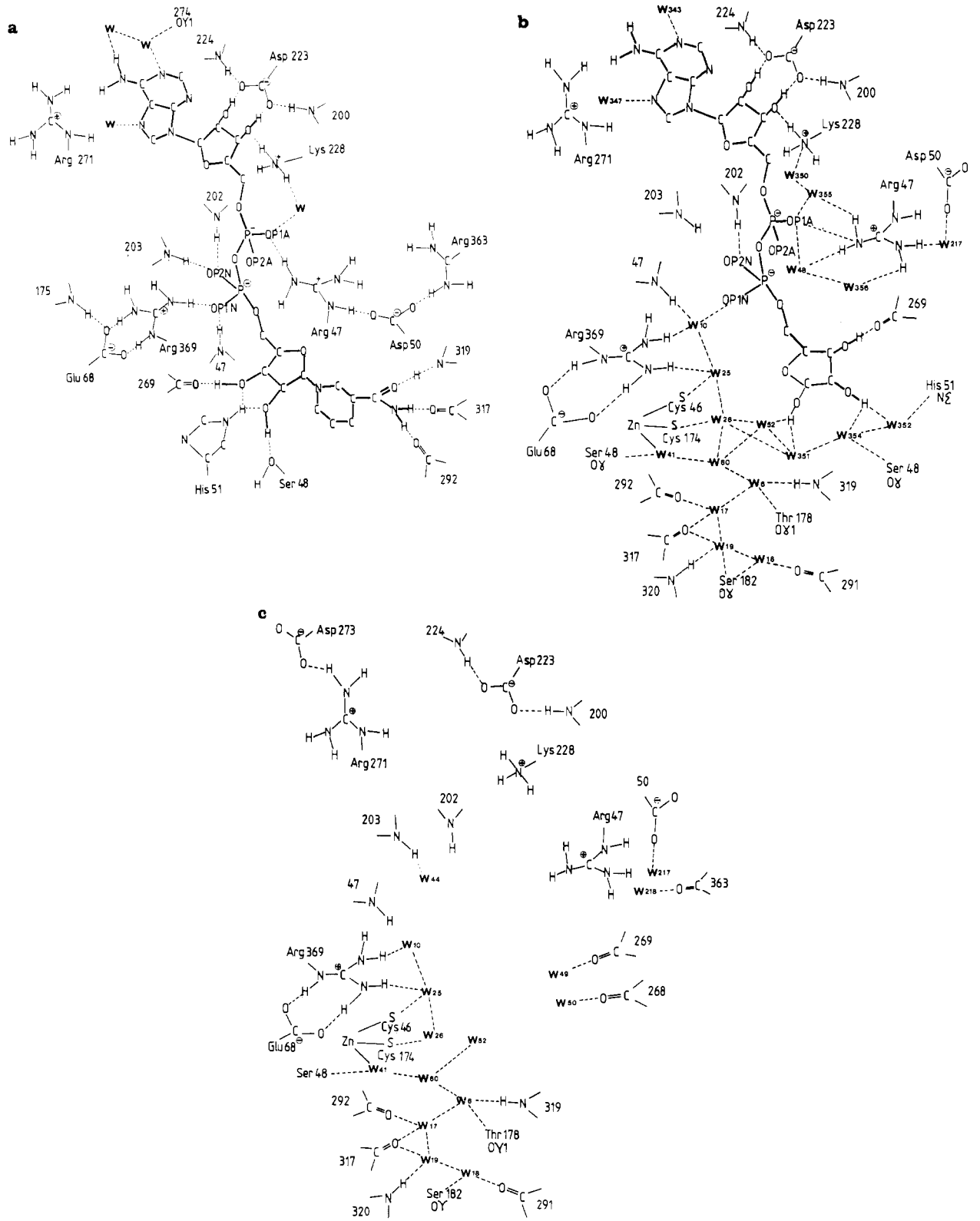

FIGURE 6: Schematic drawings showing hydrogen bonds between the enzyme and (a) NADH and (b) ADP-ribose. As a comparison, the enzyme–water hydrogen bonds in the apoenzyme are shown in (c). Hydrogen bonds are drawn as dashed lines and are assigned if the distance between hydrogen bond acceptor and donor is less than 3.3 Å. Water molecules are labeled with W. Water molecules in the ADP-ribose complex not present in the apoenzyme have numbers higher than 300. Water molecules also present in the apoenzyme have lower numbers. In the apoenzyme, W52 and W60 are not normal secondary and tertiary water molecules since they are in a deep cleft and not on the outer surface. The charged groups of residues Arg-47, Asp-223, Lys-228, Arg-271, and Asp-273 are accessible to water molecules in the apoenzyme, but we have conservatively refrained from including low-occupancy waters in the drawing.

which has the consequence that the enzyme has lost its ability to bind NAD (Thatcher & Retzios, 1980).

The site is dominated by Asp-223, which together with the glycine residues are the only structurally conserved residues between different dehydrogenases (Rossmann et al., 1975). The side chain of Asp-223 forms hydrogen bonds to the O2′A and O3′A oxygen atoms of the ribose and to the nitrogen main chain atom of residues 200 and 224. The hydrogen bonding pattern is shown schematically in Figure 6. Glu-50 in glutathione reductase interacts with the FAD coenzyme in a very

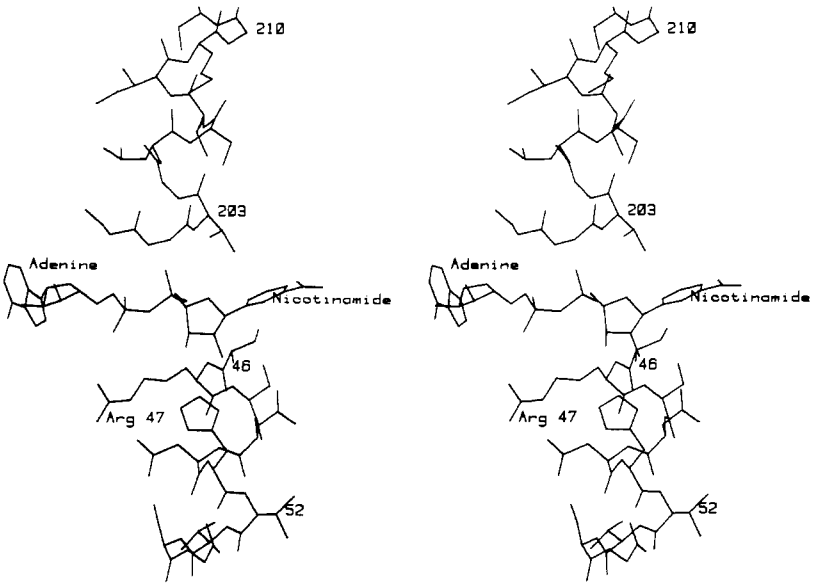

FIGURE 7: Coenzyme is bound at the amino end of two helices: α_1 from the catalytic domain (residues 47–54) and αB from the coenzyme binding domain (residues 202–214).

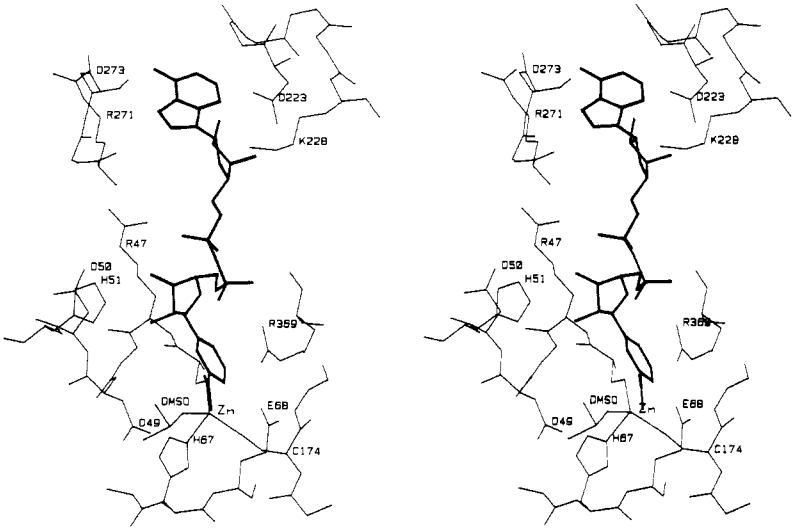

FIGURE 8: Binding site for NAD in liver alcohol dehydrogenase is surrounded by a number of charged residues. In the stereodiagram, we show these together with the residues that interact with them. The active-site zinc atom with its protein ligands and the bound inhibitor Me_2SO are also plotted. The coenzyme molecule is drawn in thick lines.

similar manner (Schulz et al., 1982). These interactions in LADH between the ribose and the protein are only possible with the ribose in the ^2E conformation. A change to ^3E conformation would bring O2′A too close to one oxygen and O3′A too far away from the other oxygen atom of the side chain of Asp-223. The binding site in LADH thus forces the sugar to adopt ^2E puckering.

O3′A is also hydrogen bonded to the side chain of lysine-228. This lysine residue is very interesting since its modification can enhance the activity of the enzyme by a factor of 10 (Plapp, 1970; Zoltobrocki et al., 1974). Structural aspects of this activation have recently been discussed from a crystallographic study of the enzyme with modified lysine residues (Plapp et al., 1983).

The van der Waals contacts between the protein and the adenosine ribose are listed in Table IIIB. The adenosine ribose has a smaller accessible surface area than the adenine (Table IV).

The adenosine binding in the ADP-ribose complex is very similar to the binding of this part of the full coenzyme. The differences in interactions seen in Table III are mainly due to the small distance variations near the cutoff limit for the table. As a consequence of the crystallographic refinement, some of the interactions of ADP-ribose with the protein differ from those described earlier (Abdallah et al., 1975; Brändén et al., 1975). Most importantly, Lys-228 makes a hydrogen bond to O3′A of the ribose and not to Asp-223.

(*C*) *Pyrophosphate Binding.* The pyrophosphate bridge of the coenzyme binds in the central part of the cleft between the domains. This binding site is situated between the amino ends of two helices: αB of the coenzyme binding domain (residues 202–215) and α1 of the catalytic domain (residues 46–52). While the axis of αB points straight at the phosphates (Eklund et al., 1981), the axis of α1 passes close to the ribose and nicotinamide (Figure 7). The pyrophosphate oxygen atoms form hydrogen bonds to main chain nitrogen atoms of residues 202 and 203 of αB and of residue 47 in the α1 helix (the latter is observed for only one subunit).

The side chains of Arg-47 and Arg-369 hydrogen bond to each phosphate of the pyrophosphate bridge and also to acidic side chains Asp-50 and Glu-68, respectively (Figure 8). The 369–68 ion pair is interior, and the glutamic acid is, in this

Table IV: Accessible Surface Areas (in Å^2) Calculated for the Atoms in the Free NADH and ADP-ribose Molecules in Our Observed Conformation and the Free Coenzyme and ADP-ribose When Bound to Alcohol Dehydrogenase[a]

	NADH in obsd conformation	NADH bound to LADH	ADPR in obsd conformation	ADP-ribose bound to LADH
N1A	22.6	4.2	22.1	6.5
C2A	46.4	0	46.5	0
N3A	20.4	0	17.3	0
C4A	7.8	0	7.0	0
C5A	7.3	0	7.5	0
C6A	10.5	0	10.6	0
N7A	23.2	7.1	25.0	6.4
C8A	29.3	15.3	32.2	18.9
N9A	0.2	0	0	0
N6A	54.4	12.9	55.1	16.1
C1′A	9.3	0	5.4	0
C2′A	11.4	10.5	10.9	10.8
O2′A	33.9	6.7	34.0	7.0
C3′A	12.3	4.4	10.5	2.8
O3′A	33.9	0.1	35.1	0
C4′A	11.6	0	16.2	0
O4′A	8.4	2.7	8.3	0
C5′A	20.9	2.7	19.5	4.7
O5′A	2.9	0.3	1.2	0
PA	1.3	0.5	2.5	0
OP1A	34.3	0.6	33.8	12.0
OP2A	33.5	0	34.6	17.0
OP3	3.5	0	5.5	0.6
PN	1.4	0	3.4	0.6
OP1N	38.6	0.1	38.7	10.3
OP2N	33.3	0	33.6	0
O5′N	0	0.1	6.2	0
C5′N	21.6	0	17.4	3.0
O4′N	8.2	0	9.5	0
C4′N	18.7	0	14.0	0.2
C3′N	14.2	0.1	17.8	4.8
O3′N	33.8	0	34.0	3.7
C2′N	7.1	0	17.6	9.1
O2′N	33.5	0	35.0	9.7
C1′N	10.0	0	23.9	0
N1N	0.1	0 (O1′N)	41.5	6.6
C2N	22.3	0.1[b]		
C3N	5.7	2.1[b]		
C4N	32.0	0.5[b]		
C5N	36.0	0		
C6N	14.9	0		
C7N	18.0	1.7[b]		
N7N	53.1	0.2[b]		
O7N	31.7	0		
	873.5[c]	72.9[c]	733.4[c]	150.8[c]

[a] As a comparison, we have calculated the corresponding free area in unbound cofactor in 5-methyl-NAD (Samama et al., 1977) to be 830 Å^2 while for NAD$^+$ with the conformation found in a Li$^+$ complex (Reddy et al., 1981) we obtained 803 Å^2. [b] These atoms have the accessible surface = 0 when substrate or inhibitor is bound. [c] Total.

case, completely buried with a hydrogen bond to a main chain nitrogen of residue 175 (Figure 6). Arg-369 and Glu-68 are conserved in all alcohol dehydrogenases. Asp-50 is at the surface of the enzyme, positioned between arginine residues 47 and 363. The carboxylic acids might play a role in orienting arginine-47 and -369 to make the most favorable specific interactions with the phosphates of the coenzyme. Dissociation of the coenzyme is the rate-determining step of alcohol oxidation. The absence of carboxylate groups would presumably enhance the arginine–phosphate interaction and therefore further slow down the dissociation. Furthermore, the ion pair Glu-68-Arg-369 allows an internal charged residue to take part in coenzyme binding.

The dipole moments of the helices make the binding site more positive. Each N-terminal helix is estimated to contribute half a positive charge (Hol et al., 1978). Similarly, Lys-228, which is the only lysine close to the coenzyme, provides an additional positive charge in the coenzyme binding site between the negative charges of Asp-223 and the pyrophosphate moiety of the coenzyme. A lysine residue, Lys-58, is present at an equivalent position in lactic acid dehydrogenase (Eventoff et al., 1977) and is conserved in all species (Eventoff et al., 1977; Hensel et al., 1983). Lys-44 has a similar position in s-MDH (Birktoft et al., 1982).

The pyrophosphate of the coenzyme is almost completely buried within the protein (Table IV); this is possible because of the polar character of the binding cleft. Only a very small part of some of the oxygen atoms is accessible to the surrounding solvent solution, and a possible water molecule can be placed between the side chain of Lys-228 and the AMP phosphate. The nonpolar interactions between the protein and the pyrophosphate (Table IIIC) involve side chain atoms of Arg-47 and also the side chains of Leu-362 and Val-203.

In the ADP-ribose complex, the interactions with residues of the coenzyme binding domain are similar, but the interactions with the catalytic domain are different. Only the side chain of Arg-47 interacts directly with a hydrogen bond to the AMP phosphate of the pyrophosphate. The closest atom of Arg-369 is 5 Å from the ADP-ribose phosphates. There is a water molecule between Arg-47 and Asp-50, and there are ordered water molecules bridging the space between the ADP-ribose molecule and the catalytic domain. The active site of the enzyme is thus connected via a water layer to the external solvent (Figures 6b and 11b).

(*D*) *NMN-ribose Binding.* The NMN-ribose binds in a narrow cleft between the two domains (Figure 9). The cleft is lined on the catalytic domain side by residues 47, 48, and 51, on the coenzyme binding domain side by residues 268–269 and 293–294, and on its inner parts by Val-203. The network of hydrogen bonds illustrated in Figure 6a involves the side chains of Ser-48 and His-51. It was suggested earlier that these residues act as a system which facilitates proton transfer from the active site to the solution. The new hydrogen bond system, which also involves the O2′N atom in the ribose, could also serve the same function (Eklund et al., 1982a).

In the ternary complexes, the NMN-ribose is almost completely buried (Table IV) while in the ADP-ribose complex the site is much more open. The ribose has fewer interactions with the protein in the ADP-ribose complex, and these interactions only occur with the coenzyme binding domain. There is only one direct hydrogen bond to the protein. O3′N is hydrogen bonded to the carbonyl oxygen of residue 269, while O2′N is hydrogen bonded via a water molecule to NE2 of His-51. O1′N also makes a hydrogen bond to a water molecule (Figure 6b). Calorimetric measurements for AMP, ADP, and ADP-ribose binding suggest that the additional ribose moiety in the latter molecule does not contribute to the binding enthalpy (Schmid et al., 1978).

(*E*) *Nicotinamide Binding.* The nicotinamide binds in a cleft in the interior of the protein, near the center of the molecule, about 15 Å from surrounding solvent. The ring interacts on one side with Thr-178, Val-203, and Val-294. The other face is directed toward the active site and is close to the catalytic zinc atom and the sulfur ligands of Cys-46 and Cys-174 (Table V and Figure 9). In all the ternary complexes we have studied, there are interactions between the nicotinamide ring and substrates or inhibitors. These interactions depend on the substrate and inhibitor and have been described elsewhere (Eklund et al., 1982a,b; Cedergren-Zeppezauer et al., 1982).

There are few van der Waals interactions between the enzyme and the nicotinamide ring. Most of these contacts involve

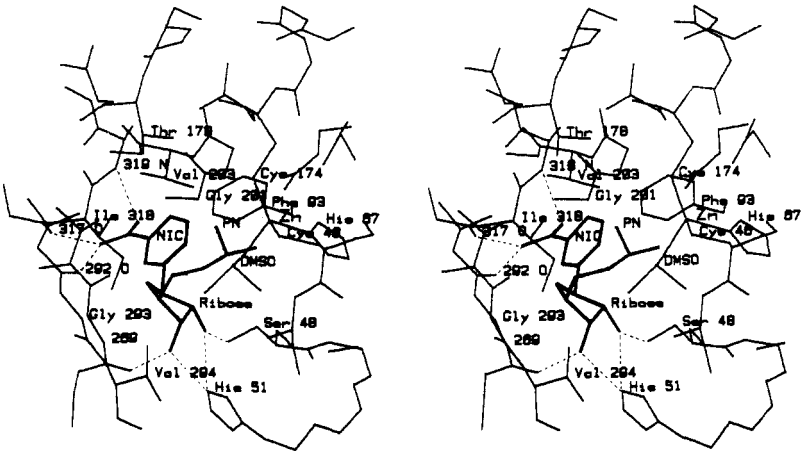

FIGURE 9: Nicotinamide binding site is located deep in the enzyme molecule. The NMN half of the coenzyme is shown in thick lines, and the neighboring protein residues, Zn, and the inhibitor Me_2SO are displayed with thin lines. Hydrogen bonds are plotted with dotted lines.

Table V: Distances from the Active-Site Zinc Atom and Its Ligands to the Four Closest Atoms of the Nicotinamide Ring of the NADH Molecule in the LADH–NADH–Me_2SO Complex[a]

enzyme atom	nicotinamide atom	distance (Å)
Zn	C4N	4.1
	C5N	3.6
	C6N	4.3
	C3N	5.1
S (Cys-46)	C4N	5.0
	C5N	3.9
	C6N	3.9
	C3N	5.9
S (Cys-174)	C4N	3.8
	C5N	3.9
	C6N	5.1
	C3N	5.1
O (Me_2SO)	C4N	3.5
	C5N	3.5
	C6N	4.1
	C3N	4.1

[a] The nitrogen atom of His-67 is further away from the nicotinamide and has not been included in the table. The distances are average values from the two subunits and differ generally by less than 0.2 Å from this average in the two subunits.

the C4 and C5 atoms (Table IIIE), which are the atoms closest to the zinc atom and its ligands (Table V).

The oxygen atom of the carboxamide is hydrogen bonded to the main chain nitrogen atom of residue 319. The nitrogen atom of the carboxamide group is hydrogen bonded to the carbonyl oxygens of residues 292 and 317 (Figure 9). The carboxamide group alone has approximately the same number of van der Waals contacts as the rest of the nicotinamide moiety.

In the structure of dihydrofolate reductase, there are three oxygen atoms that are close to and in the plane of the nicotinamide ring. Filman et al. (1982) have recently suggested that these oxygen atoms stabilize nicotinamide ring intermediates during catalysis. Alcohol dehydrogenase also has three oxygen atoms close to the nicotinamide ring: the two carbonyl oxygen atoms that hydrogen bond to the carboxamide nitrogen and the side chain oxygen of Thr-178. These oxygen atoms are all approximately 2 Å out of the plane of the nicotinamide ring. Two of these are in van der Waals contact: carbonyl oxygen of residue 292 to C2N and OG1 of Thr-178 to C4N. We can conclude that any stabilization of NAD^+ by these oxygens will be less in alcohol dehydrogenase than in dihydrofolate reductase. The effect of the carboxamide group being out of the plane improves hydrogen bond geometry and avoids too close contacts between the N7N and C2N and between the carbonyl oxygen of residue 292 and C2N. The threonine side chain is positioned at an important position in van der Waals contact to the C4 atom of the nicotinamide ring. The oxygen atom lies in the direction of the hydrogen atom of C4 on the B side of NADH. Thr-178 is conserved in all known alcohol dehydrogenases (Jörnvall et al., 1978; Dennis et al., 1984).

The role of the active-site zinc atom in NADH binding has recently been investigated by crystallographic methods (Schneider et al., 1983). This study of the active-site metal-depleted enzyme shows that the enzyme undergoes the same conformational change and that the coenzyme has the same conformation as in the native enzyme. An obvious conclusion of this is that the zinc and its ligands do not determine the conformation of the coenzyme, not even its NMN moiety. The removal of the zinc atom and the subsequent small rearrangement of the former zinc ligands have minor effects on the nicotinamide position. The tight fit of the ribose and the nicotinamide with the enzyme gives the ring little flexibility. It seems probable, therefore that the substrate rather than the nicotinamide ring has the required positional flexibility to adopt an sp^2 or an sp^3 hybridized carbon in the conversion of the substrate.

The positively charged nicotinamide ring in NAD^+ needs a compensating charge when positioned in the active site. The zinc–protein–ligand complex has a net charge of zero since the two negatively charged cysteine ligands compensate the two positive charges of the zinc ion. The introduction of the positively charged NAD^+ influences the zinc-bound nonprotein ligand in such a way that its pK value is lowered and the formation of the compensating negative charge is facilitated. NAD^+ thus causes the formation of a hydroxyl or alcoholate ion bound to the zinc atom which seems to be an important part of the mechanism of action of the enzyme (Kvassmann et al., 1981; Eklund & Brändén, 1983).

The side chain of the zinc ligand His-67 is bound to Asp-49. This negatively charged residue is too far from the nicotinamide ring to compensate the positive charge of NAD^+. It is 10 Å from the closest atom in the side chain of Asp-49 to the center of the nicotinamide ring with the Zn atom between the Asp and the nicotinamide ring.

(*F*) *Coenzyme Is A Specific.* One of the classical aspects of coenzyme binding to alcohol dehydrogenase is the A stereospecificity of the coenzyme. This was first demonstrated for the yeast enzyme (Fisher et al., 1953) and later for the liver enzyme (Levy & Vennesland, 1957). A number of

reasons for this specificity have been proposed [see Popjak (1970), Benner (1982), and Nambiar et al. (1983) and references cited in these papers]. From a structural standpoint, it is obvious that there is a steric hindrance that selects this stereospecificity. The enzyme positions both the nicotinamide ring and the substrate in a narrow pocket deep inside the protein. In the syn conformation, there is no space in the pocket to accommodate the nicotinamide ring if it is rotated 180° around the glycosidic bond. Such a change keeps the C4 of the nicotinamide ring in the same position but turns the B side toward the substrate. However, the carboxamide group would then collide with protein residues 178 and 203 and the zinc sphere, especially with the sulfur atoms of Cys-46 and Cys-174. Binding of the coenzyme to liver alcohol dehydrogenase with its B-specific side directed toward the active-site zinc atom is possible, however, for the open apoenzyme conformation of the enzyme. This has been shown in a complex where a zinc-bound imidazole molecule occupies part of the normal nicotinamide binding site (Cedergren-Zeppezauer, 1983).

Coenzyme Binding and the Conformational Change. The cleft between the domains is open in the apoenzyme form, which is well suited for the initial coenzyme binding and does not hinder its entrance. The closed form, on the other hand, would not allow the nicotinamide to enter the active site. The binding site for the AMP part of the molecule at the edge of the coenzyme binding domain is already present in the open form of the enzyme. The adenosine binding site must thus be the recognition site for coenzyme binding. Coenzyme analogues with modified nicotinamides have very similar AMP–enzyme interactions (Samama et al., 1977, 1981; Cedergren-Zeppezauer et al., 1982). However, modifications of the AMP portion drastically affect the on-velocity of the coenzyme (J.-P. Samama & H. Eklund, unpublished results).

The binding site for the remaining part of the coenzyme cannot be fully formed until the conformational change has occurred. The direct hydrogen bonds between Ser-48, His-51, and the NMN-ribose are an effect of the conformational change. This brings the side chain of Arg-369 and the pyrophosphate bridge close enough to each other to make a hydrogen bond. Residues 317–319, that form hydrogen bonds to the carboxamide, are part of the hinge region which is not affected much by the conformational change (Eklund et al., 1981).

The coenzyme is essential for inducing the conformational change of the enzyme; a structure in the closed conformation has never been observed in the absence of coenzyme. Since the ADP-ribose part of the coenzyme is not enough to produce the large conformational change, the differences between the two structures should give an insight into what causes this conformational change. We have compared ADP-ribose and NAD binding by superimposing the two structures. To obtain the structurally most relevant overlap of the two structures, the C_α atoms of both coenzyme binding domains together must be used. The structural refinement has revealed that besides the large rigid-body rotation of the catalytic domain there are small but significant conformational changes of the coenzyme binding domains. This can be described as a rotation of the domain by 1.5° around an axis across the strands of the pleated sheet. The coenzyme binding domains move in opposite directions toward the catalytic domain in each subunit (Figure 10). NAD rotates together with the coenzyme binding domain, maintaining the same interactions. The net result is that NAD is pushed deeper into the enzyme. This effect can be seen in Figure 11d, where residues at the edge of the coenzyme

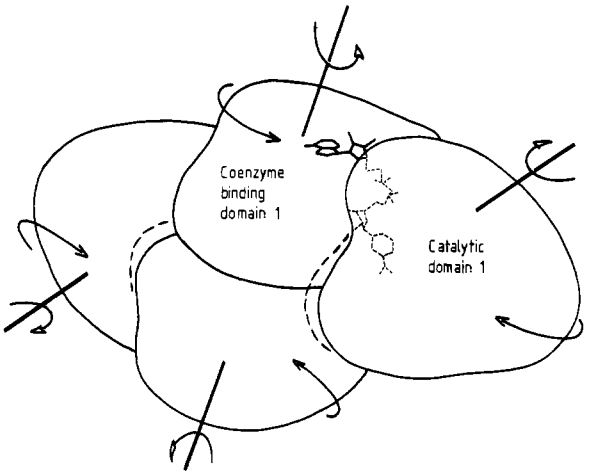

FIGURE 10: Schematic drawing showing the conformational change of the enzyme. The domains in each subunit rotate against each other. The catalytic domain rotates 10° around an axis through the domain (Colonna et al., 1984), while the coenzyme binding domain rotates 1.5° through an axis approximately across the pleated sheet strands. The coenzyme parts covered by the domain are drawn with dashed lines.

binding domain, 223, 224, and 228 move with the adenosine. The catalytic domain moves in the opposite direction, but the movements are much larger for residues far from the rotation axis, e.g., His-51. Notice that the formation of a hydrogen bond between Arg-369 and OP2N is largely due to the movement of the phosphate groups of NAD rather than the movement of Arg-369.

The coenzyme binding cleft is filled with water molecules in the absence of coenzyme (Figure 11a). We have placed about 300 well-ordered water molecules over the surface of each apoenzyme subunit. Of these, about 40 are in the clefts between the domains where the coenzyme and substrate bind. Most of the groups of the protein, which are involved in hydrogen bonds to the coenzyme, make hydrogen bonds to ordered water molecules in the apoenzyme or are accessible to bulk solvent (Figure 6c). Some of the solvent molecules are displaced when the ADP-ribose molecule binds, and a few new water interactions to the ADP-ribose molecule are found (Figure 6b). In the ternary complexes, all water molecules are displaced, and only a few water molecules are in hydrogen bond distance to the coenzyme molecule at the external solvent interface. This means that about 25 water molecules are displaced from the interior of the coenzyme binding cleft as a result of the conformational change and coenzyme binding.

After the conformational change, water molecules cannot pass from the surrounding solvent to the active site through the binding site between the domains in the closed structure. There are no channels for water in the vicinity of the bound coenzyme; the only open connection to the active site is through the substrate channel.

A quantitative measure of the effects of the conformational change can be obtained from the accessible surface areas (Lee & Richards, 1971) for the different conformations of the enzyme (Table VI). Very few atoms of the coenzyme are accessible after being bound to the enzyme (Table IV), and only about 8% of the total accessible surface of a coenzyme in the same conformation is accessible after binding to alcohol dehydrogenase. The total area of the coenzyme and the enzyme that becomes buried when the coenzyme binds is similar to the values calculated by Janin & Chothia (1978) for LDH and GPDH. It was estimated that a buried area of this size should be of the right order of magnitude to compensate for the loss of entropy during ligand binding. For LADH, the

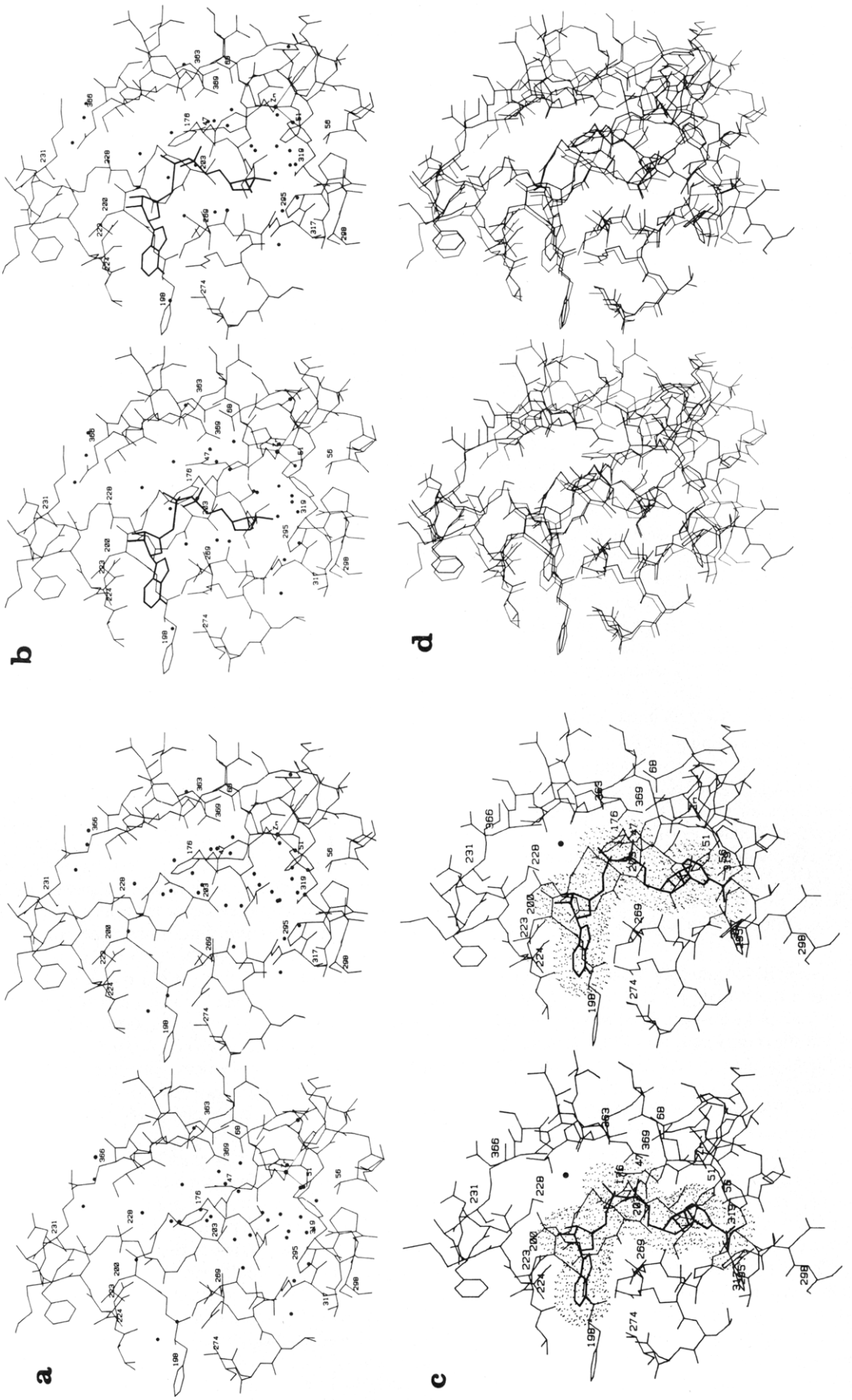

FIGURE 11: Cleft between the coenzyme binding domain and the catalytic domain in (a) the apoenzyme, (b) the ADP-ribose complex, and (c) the ternary complex with NADH and Me_2SO. Water positions in the domain–domain clefts 10 Å from OP3 of the coenzyme are shown as dots. (d) Comparison of the binding of ADP-ribose (thin lines) and NADH (thick lines) to LADH in the open and closed forms of the enzyme, respectively. The α-carbons of both coenzyme binding domains of the dimer have been superimposed.

Table VI: Surface Areas for Liver Alcohol Dehydrogenase

	Accessible Surface Areas		
	total area (Å²)	difference with apoenzyme Å²	%
apoenzyme dimer	28 240		
ADP-ribose binary complex dimer	27 651	−589	2.1
holoenzyme dimer without NADH	27 747	−493	1.7
holoenzyme dimer with NADH	26 994	−1246	4.4

Surface Areas Buried in the Ternary Complex[a]			
	LADH	LDH	GPDH
coenzyme	801	775	631
protein	450	447	433
total	1251	1222	1064

[a] Surface areas buried in the ternary complexes (Å²/subunit) calculated for LADH as described by Janin & Chothia (1978) for LDH and GPDH.

conformational change contributes an additional 247 Å/subunit.

The different energy terms involved in coenzyme binding have been calculated from microcalorimetric measurements for NAD^+, NADH, ADP-ribose, ADP, and AMP (Subramanian & Ross, 1977, 1978; Schmid et al., 1978). The normal situation for the dehydrogenases is that binding of NAD^+ and NADH has large negative enthalpy and entropy terms typical of enthalpy–entropy compensation. This is also true for ADP-ribose binding to liver alcohol dehydrogenase. However, the binding of oxidized or reduced coenzyme gives an enthalpy term close to zero and a large entropy term. The large conformational change of the enzyme must be responsible for this difference.

A necessity for the rearrangement of the domains during the conformational change is the large movement observed of the loop region of residues 292–298 of the coenzyme binding domain (Eklund et al., 1981). This part of the structure acts as a wedge between the domains; when it moves, the catalytic domain can rotate closer to the coenzyme binding domain. Therefore, the change of conformation of residues 292–298 must be an early step in the transition from the apoenzyme conformation to the ternary complex conformation. There are few contacts between the region 292–298 and the coenzyme, and we can deduce no obvious mechanisms based on the formation of charged interactions or an extensive hydrogen bond pattern. The hydrogen bond formed between the carbonyl oxygen of residue 292 and the carboxamide group may be one minor component. Besides this hydrogen bond, there are only a few van der Waals contacts between the coenzyme and Val-292 and Val-294. Thus, there is no strong driving force in the interactions between the protein loop and the coenzyme.

An indirect mechanism is probably present. The enzyme may exist in both conformations at equilibrium in the absence of coenzyme, but the equilibrium favors the open conformation. When coenzyme is bound, it stabilizes the closed conformation, and the equilibrium is shifted. The reduced coenzyme stabilizes the closed conformation to such an extent that the dissociation of reduced coenzyme, which by necessity is coupled to opening up of the coenzyme binding cleft, is rate limiting for turnover with alcohol. The opening step in this process seems in fact to be the rate-determining part of this reaction (Czeisler & Hollis, 1973). The oxidized coenzyme cannot stabilize the closed conformation to the same extent because of the positively charged nicotinamide ring.

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Registry No. LADH, 9031-72-5; NAD, 53-84-9; NADH, 58-68-4; ADP-ribose, 20762-30-5; dimethyl sulfoxide, 67-68-5.

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