The Contribution of Electrostatic and van der Waals Interactions to the Stereospecificity of the Reaction Catalyzed by Lactate Dehydrogenase

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ABSTRACT Continuum electrostatic calculations in conjunction with molecular dynamics simulations have been used to investigate the source of the stereospecificity in the hydride transfer reaction catalyzed by lactate dehydrogenase (LDH). These studies show that favorable electrostatic interactions between the carboxamide group of the reduced nicotinamide adenine dinucleotide coenzyme and protein residues of the active site of LDH can account for much if not all of the stereospecificity of the LDH-catalyzed reaction, with A-side hydride transfer more than 10^7 times greater than B-side transfer. Unfavorable steric interactions within the binding complex for B-side transfer are not found.

INTRODUCTION

Lactate dehydrogenase (LDH) catalyzes the oxidation of lactate to pyruvate by direct transfer of a hydride ion from the C2 carbon of lactate to the C4 carbon of the nicotinamide ring of oxidized nicotinamide adenine dinucleotide (NAD^+). All dehydrogenases display a marked stereospecifity, where A-type enzymes catalyze hydride transfer to and from the *re* face of the nicotinamide ring, whereas the B-type enzymes transfer to and from the *si* face. LaReau and Anderson (1989, 1992), using tritiated substrates under conditions in which the back-and-forth reaction was allowed to proceed $\sim 10^5$ times, were able to determine only a lower limit for the stereospecificity of the pig heart LDH-catalyzed reaction. Thus the transfer of the hydride to and from the *re* face of the nicotinamide ring of NAD^+ and reduced nicotinamide adenine dinucleotide (NADH) proceeds with a remarkable specificity, exceeding one part in 10^7. This requires a discrimination of over 10.4 kcal/mol favoring hydride transfer from the *re* face compared to the *si* face.

Two different mechanisms can produce the specificity of the dehydrogenases. One is catalytic specificity in which NADH could be bound with either the *re* or *si* face pointing toward substrate with roughly equal probability, but the reaction would only occur from the *re* face. Alternatively, catalysis would not distinguish the *re* and *si* faces, but rather geometrical considerations would dictate, so that the cofactor would be bound in only one conformation. Considerable effort has gone into understanding how much each of these two mechanisms contributes to the high degree of stereospecificity (Almarsson and Bruice, 1993; LaReau and Anderson, 1992; Nambiar et al., 1983; Oppenheimer, 1986; Wu and Houk, 1991; Deng et al., 1992b). X-ray crystallography has shown that pig heart and other LDHs bind NADH in the *anti* conformation about the nicotinamide glycosidic bond, with the *re* face of the nicotinamide ring pointing toward the substrate (Chandrasekhar et al., 1973). The orientation of NADH in the crystal structure suggests that binding specificity could be responsible for the catalytic stereospecificity. However, if binding plays the dominent role, then LDH must bind NADH 10.4 kcal/mol more favorably in the *anti* conformation than the *syn* conformation. The only source for the difference in binding is the carboxamide group of NAD(H), as this is the only molecular factor that distinquishes the *syn* from *anti* conformations.

Raman difference spectroscopy of NADH and NAD^+ binding to LDH from pig heart (Deng et al., 1989) provides evidence for strong interactions between the carboxamide group of NADH and the enzyme. By observing the shifts in the stretching frequency of the carboxamide carbonyl of NAD^+ and the rocking motion of the carboxamide NH_2 group of NADH upon binding LDH, Deng et al. (1991) estimated the enthalpy of interaction between the carboxamide group of the coenzyme and LDH to be 9–11 kcal/mol (Deng and Callender, 1993; Zheng et al., 1993). This may be due either to specific hydrogen bonding or to longer range interaction of the carboxamide group's dipole with the field of the protein. The magnitude of the enthalpy terms suggests that electrostatic interactions are sufficiently large to explain the stereospecific nature of LDH if these are lacking in the *syn* conformation.

That a major role for electrostatic interactions in determining binding specificity is likely is also indicated by a preliminary molecular dynamics study (LaReau and Anderson, 1992). That molecular dynamics (MD) simulation was undertaken to determine whether steric hindrance could prevent the coenzyme from binding in the *syn* conformation. No significant steric clashes were observed. However, the x-ray crystal structure that was used may have been predisposed to accept the coenzyme in the *syn* conformation, because the enzyme was crystallized with a NADH analog, (3*S*)-5-(3-carboxy-3-hydroxypropyl)-NAD^+. The

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substituent of this NADH analog occupies the space that the carboxamide group of NADH would fill if bound in the *syn* conformation. Even though LaReau and Anderson (1992) did not carry out detailed electrostatics calculations, they suggested that the interaction between the carboxamide carbonyl dipole and the α2F helix dipole is responsible for binding NADH in the *anti* conformation and, thus, the stereospecificity of the enzyme-catalyzed reaction. Detailed electrostatics calculations of NADH and NAD^+ binding to LDH (Gelpi et al., 1993), in agreement with our current findings, showed that although this particular dipole-dipole interaction contributes to coenzyme binding, it is not the main source of binding the NADH carboxamide group to LDH.

Thus many questions remain regarding the molecular source of the stereospecificity of LDH, including 1) Are steric or electrostatic interactions more important in determining the orientation of binding NADH to LDH? 2) What is the relative importance of surrounding residues for binding the carboxamide group in the *anti* conformation? 3) What possible interactions can be made between the enzyme and the carboxamide group in the *syn* conformation? 4) Which residues are responsible for the electrostatic interactions observed in the Raman experiments?

The approach we have taken to answer these questions is to directly compare the binding of NADH to LDH in the *anti* and *syn* conformations by means of detailed continuum electrostatic calculations. Restrained molecular dynamics simulations were used to generate an appropriate set of enzyme complexes for analysis (Brünger, 1992). The electrostatic interaction energies were calculated by the program DelPhi (Klapper et al., 1986; Gilson et al., 1987; Gilson and Honig, 1988; Nicholls and Honig, 1991). The overall binding free energy is the difference between the interaction free energy of cofactor with water and with the protein. Calculation of binding energies therefore requires a comprehensive treatment of the entire cofactor in both water and protein. This will not be attempted in the present study. Our goal is to determine the difference in the electrostatic and van der Waals contributions to the binding energy of the coenzyme in the *anti* versus *syn* conformation. This simplifies the problem considerably, because many factors that contribute to binding can now be taken as constant, because they will be roughly equal for NADH binding in the two conformations. For example, the interactions of the non-nicotinamide portions of the cofactor are invariant, as this is how NADH interacts with water. Our study will focus on the binding of the NADH carboxamide group to the enzyme, because this is the determinant of the difference in coenzyme binding in the *anti* versus *syn* conformation.

MATERIALS AND METHODS

Coordinates

The following x-ray crystal structures were taken from the Brookhaven Protein Data Bank (Bernstein et al., 1977; Abola et al., 1987): 1) pig M4 lactate dehydrogenase binary complex with NADH (9LDB) (Dunn et al., 1991), 2) pig M4 lactate dehydrogenase ternary complex with NADH and oxamate (9LDT) (Dunn et al., 1991), and 3) dogfish muscle lactate dehydrogenase apo-enzyme (6LDH) (Abad-Zapatero et al., 1987). The A subunit of the pig muscle enzyme structure (9LDT), which has NADH and oxamate in the active site, was used as the ternary complex. Only the active-site waters (HOH 1 to 23) were retained. The inhibitor oxamate was changed to the substrate pyruvate by making the appropriate atomic substitutions. The catalytically important active site loop (residues 98–110), which closes upon substrate binding, is closed in the pig LDH ternary complex (9LDT), nearly so in the pig LDH binary complex (9LDB), and open in the dogfish muscle apo-LDH (6LDH). A binary complex with an open active-site loop was constructed using the dogfish A subunit and the position of NAD found in the pig binary complex. The active-site residues of the dogfish muscle apo-LDH and the pig LDH binary complex were superimposed. Then the NADH coenzyme and 23 active-site waters were transferred from the pig binary structure to the active site of the dogfish apo-LDH structure.

Molecular dynamics

Molecular dynamics and energy minimization were used to generate the following four LDH complexes: dogfish muscle LDH binary complexes with NADH bound in *anti* and *syn* conformations and pig muscle LDH ternary complexes with NADH again bound in the *anti* and *syn* conformations. In the binary structures the active-site loop is in the "open" state, whereas it is "closed" over the active site in the ternary structures.

X-PLOR V3.1 (Brünger, 1992) was run on a Silicon Graphics Indigo workstation using the parameter file parhcsdx.pro and the topology file tophcsdx.pro. The hydrogen bonding and nonbonding cutoff values were set to 4.5 Å and 11.0 Å, respectively. A distant dependent dielectric constant equal to $\epsilon' R$ was used with ϵ' set to 2/Å. The ionizable residues Arg, Asp, Glu, Lys, and His were all charged, with the following exceptions: 1) The active-site His (residue 195) was uncharged, with the proton on ND1, in the binary LDH complex for both MD simulations and electrostatics calculations. This allows comparison of the computed results with the Raman vibrational data taken at pH's at which this His is expected to be neutral (Clarke et al., 1988). 2) The total charge on the active-site loop Arg (residue 101) was set to zero in the binary complex MD simulation with NADH bound in the *syn* conformation. This was necessary because a strong electrostatic interaction with the carboxamide group exaggerated the effect of this Arg. In the "open" loop the Arg is expected to be well solvated, an effect that is ignored in the MD simulatons. The Arg is positively charged in the electrostatics calculations.

The initial binary and ternary PDB structures with NADH bound in the *anti* conformation were subjected to 40 steps of Powell energy minimization after addition of the polar hydrogens, keeping all heavy atoms fixed. This was followed by 100 steps of Powell energy minimization in which all atoms were allowed to move. Additional reiterations did not yield any significant differences in the structure. The energy-minimized structures were subjected to molecular dynamics simulations consisting of a heating phase of 0.2 ps to a final temperature of 300 K, an equilibration phase of 0.2 ps, and an observation phase of 10 ps. A time step of 1 fs was used. In the MD simulations only residues with atoms within 10 Å of the C4N atom of NADH were free to move. The oxygen atoms of the 23 active-site waters were constrained to their original positions by using a harmonic potential with a weighting constant of 20 kcal/mol/Å^2. Furthermore, the C4N (NADH)-C2 (pyruvate) distance in the ternary complex was constrained to its initial value of 3.5 Å by using a biharmonic potential to ensure that the final structure would correspond to an active enzyme complex.

The dihedral angle for the nicotinamide glycosidic bond of *anti* NADH, defined by the O4'N, C1'N, N1N, and C2N atoms, was constrained to its initial value (−101.9° for the binary structure and −94.3° for the ternary structure) by a harmonic energy constant of 200 kcal/mol/rad^2 during all molecular dynamics and energy minimization runs. The LDH complexes with NADH bound in the *syn* conformation were generated by taking the appropriate final *anti* structure, rotating the nicotinamide by 180° around

the glycosidic bond, and repeating the energy minimization and MD procedure outlined above. The glycosidic bond dihedral angles were now restrained to 78.7° and 84.9° for the binary and ternary complexes, respectively. To accommodate the carboxamide group of NADH in the active site in the *syn* conformation, three active-site structural water molecules were removed (H_2O- 8, 9, 10). As before, the MD simulations were followed by 100 steps of Powell energy minimization to yield a final structure for electrostatic and van der Waals analysis.

Electrostatics

Continuum electrostatics calculations were carried out with the program DelPhi, which uses the finite-difference method to solve the Poisson-Boltzmann equation (Klapper et al., 1986). The protein was mapped onto a 65^3 cubic grid. Electrostatic potentials were calculated with progressively increased resolution, from an initial grid spacing of 0.3 grids·Å^{-1} to a final grid spacing of 2.4 grids·Å^{-1} centered on the C7N atom of NADH, using the focusing method (Gilson et al., 1987). The dielectric constant within the protein was set to 2, and outside the protein it was set to 80. The protein molecular surface, which defines the dielectric boundary, was assigned using a 1.4 Å water probe radius. The solvent ionic strength was zero.

Electrostatic interaction energies between the carboxamide group of NADH and the LDH protein were determined from the electrostatic potential generated by the carboxamide atomic partial charges at the LDH atomic sites. The carboxamide atomic partial charges were taken from the X-PLOR topology file topology.nad (C, +0.38; O, −0.38; N, −0.83; H, +0.415). Atomic partial charges for the protein residues were taken from the CHARMM force field. TIP3 charges were used for the 23 active-site structural waters.

The interaction between the carboxamide -C═O (or $-NH_2$) and the specific groups in the protein is determined by a DelPhi calculation in which only the atoms of the -C═O (or $-NH_2$) dipole have any charges (Gilson and Honig, 1988). DelPhi then provides the potential at all other atoms in the protein. The interaction energy between the dipole and any protein atom is the product of the potential at that atom from the -C═O (or $-NH_2$) dipole times the partial charge on that protein atom. Residue interaction energies represent the sum over the atoms in the residue of the atomic interaction energies. The interaction of a residue with the carboxamide is the sum of the interactions with -C═O and $-NH_2$.

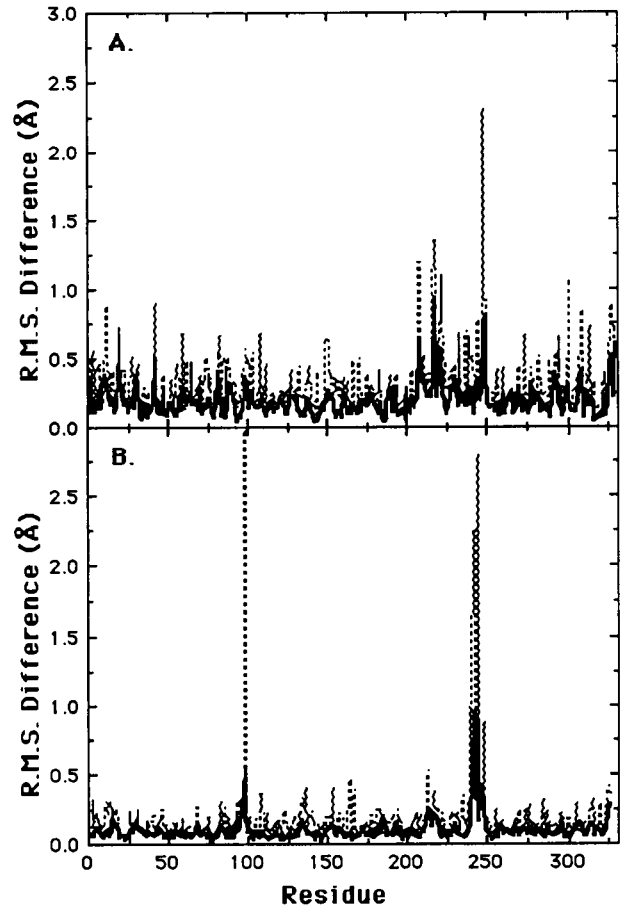

FIGURE 1 The average difference in position (root mean square deviation) of each protein residue, averaged over the side-chain (– – –) or backbone atoms (——), for protein with NADH bound in the *syn* versus *anti* conformation in (*A*) the binary complex and (*B*) the ternary complex.

RESULTS

Molecular dynamics

The binary complexes were built by docking the NADH from the binary pig LDH into the dogfish muscle apo-LDH as described in Materials and Methods. This provided a complex that is not biased toward the *syn* or *anti* conformation because of the contacts in the crystalized protein. In addition, the loop is fully open in the dogfish apo-protein, whereas it is partially closed in the pig binary complex (Abad-Zapatero et al., 1987). The results of the binary complex may provide information about the initial docking interactions between protein and cofactor, before the loop closure that traps the NADH. In this regard it is interesting that binding the *syn* or *anti* NADH into the apoprotein requires very little modification of the protein structure. The heavy atom rms deviation is less than 0.5 Å. Only the side chains of Arg-101 and Asn-138 move in response to the introduction of cofactor.

Fig. 1, *A* and *B*, shows the average change in position (root mean square deviation) of each protein residue, averaged over the sidechain or backbone atoms, for NADH bound in the *syn* conformation with respect to the same complexes with NADH bound in the *anti* conformation for the binary and ternary complexes, respectively. No large-scale protein rearrangements are necessary to accommodate the carboxamide group of NADH after rotating the nicotinamide ring 180° about the glycosidic bond. In both the binary and ternary complexes only a few protein residues undergo significant movement during the molecular dynamics simulation. The only steric interaction that must be relieved in the MD simulation for both the binary and ternary enzyme complexes with NADH bound in the *syn* conformation is between Thr-246 and the carboxamide group. Movement of other protein residues in the MD simulation, such as Arg-101 in the ternary system, are the result of unfavorable electrostatic interactions.

NADH binds in a cleft in the protein. The carboxamide faces into the cleft in the *anti* and toward the solvent in the *syn* complexes (LaReau and Anderson, 1992). Thus, whereas none of the carboxamide is solvent exposed in either *anti* complex (probe radius 1.4 Å), in the binary *syn* structure, 11 Å^2 of the carbonyl oxygen and 6 Å^2 of the amine nitrogen are exposed to water. Thus there is no real

posibility for steric hinderence providing the predominant force disfavoring the NADH binding in the *syn* binary conformation. In the *syn* ternary complex only 0.5 Å of the oxygen and none of the amine is now solvent accessible. However, closed-loop ternary complexes can be formed with NADH in the *syn* conformation. In all cases, the carboxamide has favorable van der Waals interactions with the protein (see Table 1); however, the *anti* conformation is slightly more favorable in both the binary and ternary complexes.

Protein motion is found to be more limited in MD simulation of the ternary complex than in the binary complex (see Fig. 1), in agreement with Raman studies of cofactor binding to LDH (Deng et al., 1992a). Loop closure (residues 98–110) over the active site upon substrate binding and the two additional charged residues in the active site of the ternary complex (His-195: +1; pyruvate: −1) may contribute to the increased rigidity. The additional charges in the active site on the pyruvate and the histidine cause other ionizable residues to be drawn closer into the active site in the MD simulation. This includes Asp-168, which forms an ion pair with the active-site histidine, and Arg-171, which is partly responsible for binding the substrate through an interaction with its carboxylate moiety.

Electrostatics

The difference in the interaction energy between the *anti* and *syn* conformations of the carboxamide group with charged and dipolar groups in the protein is −12.3 kcal/mol in the binary and −8.8 kcal/mol in the ternary complex (see Table 1). The orientation of the carboxamide into the binding cleft in the *anti* complex and toward the solvent in the *syn* complex influences the reaction field (or solvation) energy of the complex. The increased contact with water stabilizes the *syn* relative to the *anti* conformation by −1.9 kcal/mol in the open-loop structure and −1.4 kcal/mol in the closed compex. Thus the total difference in electrotatic interaction of the carboxamide with the protein and water of −10.4 and −7.4 kcal/mol in the binary and ternary complexes, respectively, is sufficient to account for the much of the observed catalytic stereospecificity of LDH.

The larger, favorable interactions between the carboxymide and protein result primarily from NADH having significantly more hydrogen bonding partners in the *anti* conformation. Thus in the binary complex the carboxamide NH_2 is hydrogen bonded to the backbone carbonyl of Val-138 and to the side chain of Asn-140, whereas the carboxamide carbonyl is hydrogen bonded to H_2O-1 (see Table 2 for hydrogen bond energies and distances). In contrast, the binary *syn* complex has close contact between the carboxamide NH_2 and Arg-101, which destabilizes NADH binding. Although the final Arg position that yields this unfavorable contact may be a result of the Arg charge being set to zero in the MD simulation, no favorable interactions are found by the cofactor in the simulation. Thus three residues make hydrogen bonds to the carboxamide in the *anti* orientation, whereas none are availible in the *syn* conformation. It would appear that the NADH will be bound preferentially in the *anti* conformation even in the open-loop, binary encounter complex.

In the ternary, *anti* complex, the hydrogen bonds between the carboxamide NH_2 and the Val-138 backbone and Asn-140 side chain are maintained (Table 3 and Fig. 2). In addition, the side chain of Ser-163 is now within hydrogen bonding distance. The hydrogen bond between the water

TABLE 1 The calculated van der Waals and electrostatic energies (in kcal/mol) of the interactions of the carboxamide of NADH with protein in the *anti* and *syn* conformations in both the binary (NADH/LDH) complex and ternary (NADH/LDH/pyruvate) complex

	van der Waals	Electrostatic charge: charge*	Electrostatic reaction field#	Total energy
Binary complex				
Anti	−6.4	−10.1	0.0	−16.5
Syn	−5.1	2.2	−1.9	−4.8
Difference	−1.3	−12.3	1.9	−11.7
Ternary complex				
Anti	−6.6	−16.2	0.0	−22.8
Syn	−4.2	−7.4	−1.4	−13.0
Difference	−2.4	−8.8	1.4	−9.8

*The interaction of the carboxamide dipole with other charges and dipoles in the protein and with buried, crystallographic waters.
#The reaction field energy is the stabilization of the carboxamide dipole by the high dielectric medium outside of the protein.

TABLE 2 The electrostatic interactions, greater than 0.6 kcal/mol, and separation between the carboxamide group of NADH with specific residues of LDH in the binary complex for the *anti* and *syn* conformations

	Interaction with NH_2	Separation (Å)§	Interaction with C=O	Separation (Å)§	Total interaction
Anti					
Val*-138	−2.2	1.9	−0.9	8.1	−3.1
Asn-140	−1.9	2.2	−0.8	3.8	−2.6
Asp-143	−0.6	8.3	−0.1	10.3	−0.7
Asp-168	0.0	6.9	0.7	6.7	0.7
H_2O-1	−0.7	3.6	−2.9	2.9	−3.5
Total#	−5.2		−4.9		−10.1¶
Syn					
Arg-101	1.3	2.1	0.8	4.5	2.2
Total#	1.0		0.9		2.0¶

All energies are in kcal/mol.
*Interaction is with the backbone amide; all other values are for interactions with side chain.
#The total is the sum of all of the electrostatic interactions between the protein and the carboxamide group, including groups not explicitly listed.
§Distances are between the hydrogen bonding protein and the heavy atom hydrogen bond acceptor.
¶His-195 is assumed to be neutral in the binary complex (see Materials and Methods). If it is protonated, the interaction energy is only −0.2 kcal/mol lower in the *syn* complex and −0.6 kcal/mol lower in the *anti* complex.

TABLE 3 The electrostatic interactions, greater than 0.6 kcal/mol, and separation between the carboxamide group of NADH with specific residues of LDH and substrate in the ternary complex for the *anti* and *syn* conformations

	Interaction with NH_2	Separation (Å)[§]	Interaction with C=O	Separation (Å)[§]	Total interaction
Anti					
Arg-109	−0.8	5.6	−0.2	5.1	−0.9
Val*-138	−2.8	1.8	−0.8	4.9	−3.6
Asn-140	−1.4	2.5	−0.4	3.6	−1.9
Ser-163	−2.0	2.1	0.0	4.0	−2.0
Asp-168	0.4	7.3	1.1	6.0	1.5
Arg-171	−0.6	8.1	−0.7	5.9	−1.2
His-195	−1.9	3.8	−2.7	2.4	−4.6
Pyruvate	1.7	5.6	1.3	3.9	3.0
H_2O-1	−0.5	2.4	−2.1	1.8	−2.6
Total[#]	−9.8		−6.4		−16.3
Syn					
Arg-101	0.3	7.2	0.3	10.0	0.6
Arg-171	−1.0	5.7	−1.0	4.2	−2.0
His-195	−0.8	7.0	0.0	6.0	−0.8
Thr-246	−2.3	1.9	−0.5	4.8	−2.7
Pyruvate	1.9	4.7	0.8	3.7	2.6
H_2O-23	−0.4	4.3	−2.4	1.9	−2.8
Total[#]	−4.0		−3.4		−7.4

All energies are in kcal/mol.

*Interaction is with the backbone amide; all other values are for interactions with side chain.

[#]The total is the sum of all of the electrostatic interactions between the protein and the carboxamide group, including groups not explicitly listed.

[§]Distances are between the hydrogen bonding protein and the heavy atom hydrogen bond acceptor.

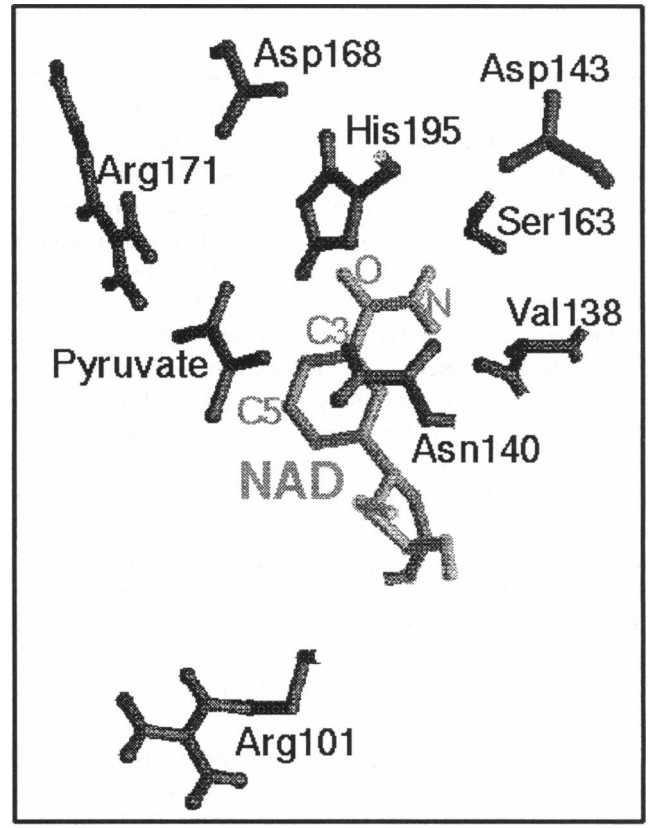

FIGURE 2 A sketch of bound cofactor and the residues from Table 3 in the ternary complex and approximate relative distances.

and the carboxamide C=O is retained. This serine and its interaction with a bound water is conserved in all known lactate dehydrogenases (Wigley et al., 1992). In addition, the active-site His-195, which is now protonated, is 2.4 Å from the carbonyl. In the closed, *syn* ternary complex NADH can now make two hydrogen bonds: its amine to the backbone carbonyl of Thr-246 and its carbonyl to water-23. Thus five residues make hydrogen bonds to the carboxamide in the *anti* orientation, whereas only two are made in the *syn* conformation. Thus both *syn* and *anti* conformations have favorable electrostatic interactions with the protein; however, these are 8.8 kcal/mol more favorable for the *anti* bound NADH (see Tables 1 and 3). The amide backbone of the α2F helix contributes only 0.45 kcal/mol to the carboxamide binding energy for the LDH/*anti*-NADH binary complex. This rules out the suggestion (LaReau and Anderson, 1992) that a dipole-dipole interaction with the α2F helix plays a major role in binding NADH in the *anti* conformation.

Closing the active-site loop (residues 98–110) acts to increase the number of hydrogen bonds between the carboxamide and the protein. In addition, the greater burial of the carboxamide allows for longer range interactions with the protein. Thus in the binary, *anti* complex there are interactions on the order of ±0.6 kcal with Asp-141 and -166, which are more than 3 Å from the carboxamide. In the *anti*, ternary complex with a closed loop, Asp-168, Arg-171, and the pyruvate substrate all have interactions with the carboxamide greater than ±1.2 kcal/mol, despite being more than 3 Å away.

DISCUSSION

The central question of this work is whether the extreme stereospecificity of the LDH-catalyzed hydride transfer reaction can be accounted for by differences in the affinity of the carboxamide group of NADH in the *anti* and *syn* conformations. The observed stereospecifity is equivalent to a 10.4 kcal/mol difference favoring transfer of the *pro-R*, *re* face over the *pro-S*, *si* face hydrogen of NADH to substrate (LaReau and Anderson, 1992). The *anti* conformation, observed in the crystal structures of LDH (cf., Grau et al., 1981), leads to transfer of the *pro*-R hydrogen of NADH, whereas the *syn* conformation, were it populated, would lead to a transfer of the pro-S hydrogen. The molecular dynamics calculations show that there is no steric hindrance to the binding of NADH in the *syn* conformation, in agreement with the previous calculations of LaReau and Anderson (1992). The difference in van der Waals interactions betwen *syn* and *anti* complexes is on the order of 2 kcal/mol (Table 1), with the *anti* conformation favored. In the *syn* complex the carboxamide faces into the solvent-accessible

region of the binding cleft, allowing room for some substituents. It is clear that steric interactions do not prohibit substituents in the *syn* position, because 5-methyl-NAD^+ and 5-(3-carboxy-2-hydroxypropyl)-NAD^+, which contain bulky groups in this position, bind readily to LDH and are active cofactors (LaReau and Anderson, 1992; and Grau et al., 1981). Added reaction field (solvation) energy favors the partially exposed *syn* carboxamide by a small amount (<2 kcal/mol). The major difference by far between the *syn* and *anti* conformers lies in the charge-charge electrostatic interaction of the carboxamide dipole with charges and dipoles of the protein. This favors the *anti* complex by −12.3 kcal/mol for the binary and −8.8 kcal/mol for the ternary complex. The large difference in electrostatic interaction energy is primarily due to the larger number of hydrogen bond partners for the carboxamide in the *anti* complex. Taking the overall electrostatics and van der Waals contributions, the *anti* complex is favored by −11.7 kcal/mol in the binary and −9.8 kcal/mol in the ternary complex. Both of these values are sufficient to account for the −10.4 kcal/mol required for the observed stereospecificity in the binary complex within the errors inherent in the analysis.

The calculations suggest an equivalent role for the C═O and -NH_2 groups in binding to the active site in the binary complex and a somewhat larger role for the -NH_2 group in the ternary complex. On the other hand, there are two pieces of evidence that suggest that, although both play an important role, the carbonyl is more important. Raman studies suggest that the hydrogen bond between the C═O group and protein is about twice as strong as that for the -NH_2 group (Deng et al., 1991). The studies of the stereospecificity for binding shows that removing the -NH_2 group and replacing it by -CH_3, a group unable to make hydrogen bonds, lowers the specificity by only 2.2 kcal/mol, about 25% of the 10.4 kcal/mol found for NADH (LaReau and Anderson, 1992). In the calculations reported here, the balance between the -C═O and -NH_2 groups depends on the partial charges on the carboxamide atoms. The values, taken from the standard XPLOR parameter set, may underestimate the partial charges associated with the C═O group and overestimate that of the -NH_2 group. In addition, because the carboxamide has zero net charge in the XPLOR charge set, there is no contribution of the carboxamide group withdrawing electrons from the pyridine ring that is known to occur (e.g., Fischer et al., 1988; Deng et al., 1991). The extra charge from the electron withdrawal would be localized primarily on the carbonyl oxygen. The interaction energies calculated with DelPhi are obtained from the product of the potential at the carboxamide from the protein and the partial charge on each carboxamide atom. Therefore, any changes in the charge distribution will have a direct effect on the interaction energies. Thus the inaccuracy of charge sets limits on the accuracy of the electrostatic calculations, and it does seem reasonable that the contribution of the carbonyl has been underestimated.

Our calculations suggest that the energy difference between the *anti* and *syn* conformers is somewhat less in the ternary complex than in the binary complex. The results of the binary complex are more relevant to the issue of stereospecificity, because there are at least two other factors, other than energy difference, that play a role in limiting hydride transfer to the pro-S hydrogen in the ternary complex. One is that NADH likely does not have time to come to equilibrium when substrate is added to the binary complex to form the ternary complex. The nicotinamide ring is not free to rotate sterically from an *anti* to a *syn* conformer in the binding cleft, so there will be a substantial barrier to moving the carboxamide from the *anti* to the *syn* conformation without release of the cofactor. This is particularly true for the ternary complex, in view of the present MD calculations and Raman measurements (Deng et al., 1992a), which show that the protein becomes more rigid in the ternary complex. In pig heart LDH, the rate-limiting step in the catalysis of substrate to product is product release, and this occurs on the millisecond time scale (k_{cat} = 250 s^{-1}; cf. Clarke et al., 1986). Given this rate, there would be no significant population depletion from the initially formed *anti* conformation to the *syn* conformer, assuming only a modest rotation barrier. A calculation based on the standard equation for reaction rates using a Boltzmann distribution that describes the thermal population of states and a preexponential factor of 10^{13} s^{-1} shows that only a 16 kcal/mol barrier is required to slow the rate to 250 s^{-1}.

The second factor that could favor reaction in the *anti* complex is that loop closure may be more difficult when NADH adopts the *syn* conformation. Substrate binding to the LDH/coenzyme binary complex induces the loop residues 98–110 to close over the active site. The conformational change yields a number of important contacts that enhance the catalytic efficiency of the enzyme. Mutation of Arg-109 to Gln, which probably prevents "loop closure" altogether, weakens K_M for pyruvate by a factor of 30-fold and decreases k_{cat} for hydride transfer by over 1200-fold (Clarke et al., 1986). Some of the catalytic enhancement arises from the specific electrostatic contact of Arg-109 with substrate. Raman difference measurements have shown that the enthalpy of the hydrogen bond between the carbonyl oxygen of the substrate and Arg-109 is about 4 kcal/mol (Deng et al., 1994), and this specific contact makes a substantial contribution to the stability of the transition state of the catalyzed reaction (Clarke et al., 1986; Deng et al., 1994). In addition, the ternary, closed complex is more rigid than the binary complex (see Results and Deng et al., 1992a), which can decrease the number of accessible, unproductive conformational states of NADH. The present calculations, as well as the enzymatic activity with hydrophobic, *syn* substituted cofactors (LaReau and Anderson, 1992), show that steric hindrance does not prohibit loop closure. Rather, the present calculations show an unfavorable electrostatic interaction between Arg-101 and the carboxamide NH_2 group in the *syn* binary complex (Table 2), as well as a large rms deviation for this Arg in the *syn*

ternary complex (see Fig. 1 *B*). This repulsive electrostatic interaction may upset the balance between the energy needed to drive loop closure and that gained from enhanced electrostatic interactions when the loop closes (see also Gelpi et al., 1993).

Finally, the absorption spectrum of the nicotinamide ring of NADH shows significant bathochromic shifts in going from an apolar solvent to a polar solvent, which can serve as a probe of the binding site or solvent polarity (Cilento et al., 1958; Fisher et al., 1969; Fischer et al., 1988). An analysis of the resonance structures contributing to the ground and first excited states suggests that the dipolar resonance structure of NADH with a π-conjugation between the dihydropyridine ring and the carboxamide substituent contributes more to the first excited state than to the ground state (Cilento et al., 1958). In polar solvents this dipolar resonance structure is stabilized, causing a reduction in the transition energy between the ground and first excited states (i.e., a red shift in the absorption spectrum). In general, A-side enzymes, like LDH, cause small (2–14 nm) blue shifts, whereas B-side enzymes result in larger (10–30 nm) red shifts (Fischer et al., 1988). This result is surprising, at first sight, given the number of hydrogen bonds formed between the surrounding protein and the carboxamide of NADH bound to LDH in our calculations. It would seem that the binding site of LDH is very polar. However, our electrostatic calculations show that, despite these specific interactions with the carboxamide group of NADH, there is not a large gradient across the π electron structure of the entire nicotinamide ring, that is, from N1 of the pyridinium ring to the carbonyl oxygen or the carboxamide. Thus it is reasonable to suppose that this particular configuration of the protein active-site potential does not stabilize the dipolar resonance structure of the niconamide ring as efficiently as the reaction field in water, and our results are in qualitative agreement with the observed relatively small blue shift in absorption when NADH binds to LDH.

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