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Catalysis by Entropic Guidance from Enzymes†

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ABSTRACT: Certain features underlying enzymatic catalysis, such as energetic stabilization from binding interactions or proximity and orientation of chemical groups, are evident in the equilibrium-averaged structure of an enzymatic complex determined by crystallography or NMR. Transient features are not apparent from an average structure. Here, we report on a catalytically relevant property of an enzymatic complex revealed by thermal fluctuations from a molecular dynamics study. The conformational fluctuations of the cofactor NADH are altered by binding the enzyme lactate dehydrogenase (LDH) compared to those of free NADH; thermal motions give rise to structures similar to that of the putative transition state. The alteration is stereospecific, in agreement with measured changes in vibrational spectra, and leads to an understanding of the correlation, established some time ago by crystallography and NMR, between the nicotinamide glycosidic bond torsion angle (*anti*/*syn*) and the stereospecificity of hydride transfer. These results suggest that one catalytic role of the enzyme is to funnel the population of NADH conformers to the transition state and reduce the entropic barrier to activation. The specific motions in an enzyme complex that might function to enhance transition state formation are described.

The catalytic power of an enzyme molecule can be understood, in part, from factors apparent in an equilibrium-averaged structure (Page, 1987). In some cases, further insight into enzymatic catalysis can be gained from knowledge of the fluctuating conformations from which the average structure is obtained (Post & Karplus, 1986). The importance of fluctuations has also been recognized for ligand binding and dissociation, as established by detailed studies of myoglobin (Case & Karplus, 1979; Elber & Karplus, 1990). With regard to catalysis, results of a recent molecular dynamics study (Young and Post, manuscript in preparation) suggest that binding of NADH to LDH alters the conformational energy of NADH such that thermal fluctuations lead to structures resembling the generally accepted transition state structure. Thus, binding LDH guides NADH toward the transition state by modulating its equilibrium conformational fluctuations and reducing the entropic barrier to activation. The entropic benefit of binding a flexible substrate or cofactor in a particular orientation that promotes the reaction is well recognized in the study of enzymatic catalysis (Page, 1987). Proposed here is a different source of entropic guidance by enzymes, beyond the selection of one of several dihedral rotamers, whereby the fluctuations associated with a single conformational minimum are altered by binding interactions to confer a catalytic advantage.

Dehydrogenases catalyze oxidation/reduction reactions through a stereospecific hydride transfer between the cofactor NADH and a substrate. LDH catalyzes the transfer of H4A (Figure 1A) to pyruvate and is therefore an A-stereospecific enzyme (LaReau & Anderson, 1992). Other dehydrogenases have H4B stereospecificity. Enzymes which catalyze hydride transfer from the A-side are known to bind the cofactor with the carboxamide of nicotinamide *anti* to the ribose, while B-stereospecific enzymes bind the *syn* conformation. The

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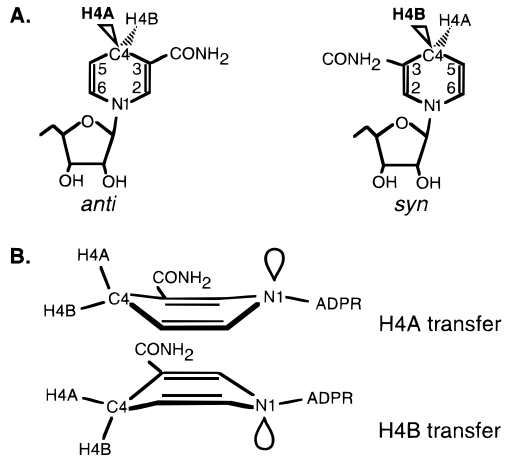

FIGURE 1: (A) Correlation between stereospecificity of hydride transfer by dehydrogenases and glycosidic bond conformation. A-stereospecific enzymes catalyze transfer of H4A and bind *anti*-NAD(H) while B-stereospecific enzymes catalyze transfer of H4B and bind *syn*-NAD(H). All structures of enzyme-bound nicotinamide cofactors determined by crystallography and NMR show the relationship between stereospecificity and glycosidic bond conformation [Table I in the reference Wu and Houk (1991); Levy *et al*., 1983)] with the possible exception of type II dihydrofolate reductase (DHFR) from a bacterial plasmid (Brito *et al*., 1991) which differs from chromosomal DHFR. The correlation does not apply for enzymes with a second coenzyme as shown by the one case of glutathione reductase which utilizes FAD (Karplus & Schulz, 1989). (B) Putative transition state for hydride transfer at C4 at dihydronicotinamide for either H4A or H4B transfer. That a boat-like conformation of the dihydronicotinamide ring facilitates hydride transfer has been discussed by a number of authors (Benner, 1982; Nambiar *et al*., 1983; Levy & Vennesland, 1957; Oppenheimer *et al*., 1978; Wu & Houk, 1987; Almarsson & Bruice, 1993). *Ab initio* studies find that the transition state is puckered (Wu & Houk, 1987). *Syn* elimination in experimental systems and the N1 lone pair electrons being on the same face as the transferring axial hydrogen are discussed by Benner (1982).

generally accepted transition state for hydride transfer includes a boat-like conformation of the dihydronicotinamide base (Benner, 1982; Nambiar *et al*., 1983; Levy & Vennesland, 1957; Oppenheimer *et al*., 1978; Wu & Houk, 1987; Almarsson & Bruice, 1993) (Figure 1B). Transfer of the axially positioned hydrogen at C4 is assisted by an axially positioned lone pair of the nitrogen, N1, so that puckering toward either the A-side or B-side of the ring favors transfer of H4A or H4B, respectively.

To investigate the influence of the enzymatic active site interactions on the conformational equilibrium of dihydronicotinamide, we compared ring puckering of dihydronicotinamide mononucleoside (NMSH) *in vacuo* to that of NADH in the solvated complex LDH−pyruvate−NADH. Free energy perturbation methods of molecular dynamics were used with stochastic boundaries and a conformational "reaction coordinate" (Tobias & Brooks, 1987, 1988) for boat-like puckering. The free energy as a function of ring puckering was calculated by constraining the geometry of only the six ring atoms while sampling all other internal degrees of freedom.

The intrinsic nature of ring puckering was assessed from *in vacuo* NMSH. The calculated free energy of puckering has a broad minimum (Young & Post, 1993); the planar ring conformation is the lowest energy conformation, but the energy cost for boat-like puckering is small. Estimation of the free energy change for puckering with N1 and C4 ±20° out of plane gives a value less than 1 kcal/mol, so that thermal fluctuations would give rise to substantial boat-like puckering in both directions.

Binding in the nucleotide site does not alter the free energy profile for puckering with respect to the six-membered ring but does affect the thermal motions associated with puckering in a manner that promotes a transition state-like structure for hydride transfer. A role of the enzyme might have been to bind the puckered conformation with H4A axially positioned. On the contrary, the free energy profiles for puckering—as defined by the six-membered dihydronicotinamide ring—are similar for NADH in the ternary LDH complex and NMSH *in vacuo*, and preferential stabilization of A-side puckering is not supported. However, the configurations of N1 in the puckered ring differ between enzyme-bound NADH and free NADH. The important observation of these studies is that thermal fluctuations about the minimum free energy planar conformation lead to structures with N1 in the configuration thought to be the reactive one, unlike the fluctuations of equivalent energy in the absence of the enzyme. Binding free energy is not used to maintain on average a nonplanar ring conformation. Instead, the confinement of the active site prevents fluctuations to conformations that occur in the free state and dictates fluctuations that produce the reactive ring structure by spatially restraining certain NADH atoms. Such enhancement of structures similar to that expected for the transition state among those populated by thermal motions would promote the reaction entropically. A restriction on the bound-ring conformations was suggested to be the basis for an observed narrowing of Raman vibrational bands of the ring in a ternary LDH complex (Deng *et al*., 1992a), although the structures involved cannot be elucidated by Raman spectroscopy. In the case here, the difference in conformations from fluctuations in ring puckering is shown in Figure 2, in which puckered forms of NADH bound to LDH (Figure 2A,B) are compared to those of NMSH *in vacuo* (Figure 2C,D). Fluctuations to A-side puckering of *bound* NADH lead to a transition state-like N1 configuration (right-hand side in Figure 2A,B) in which the ribose is pseudoequatorial and N1 is pyramidalized with the lone pair electrons predominantly on the same side of the ring as H4A, or pseudoaxial, and favorable for hydride transfer. On the other hand, neither B-side puckering of the bound NADH (left-hand side in Figure 2A,B) nor *in vacuo* NMSH puckering in either direction (Figure 2C,D) leads to such a conformation; these fluctuations give rise to a pseudoaxial position of the ribose with N1 pyramidalized opposite in direction to that in the transition state model. The distinction of A-side puckering in enzyme-bound NADH from the other three conformers in Figure 2 is consistent with measured vibrational frequencies of NADH free and bound to LDH (Deng *et al*., 1992b); the frequency of the carbon−hydrogen stretching mode is nearly the same for the two C4−hydrogen modes of free NADH, while one mode of the binary LDH−NADH complex shifts upward and the second mode is relatively unchanged. The differential shift for the two modes in the binary complex could reflect the conformational asymmetry of puckering in the enzyme active site.

The essential element for directing the puckering motions toward a reactive ring conformation is the configuration of N1. In the absence of enzyme, N1 is pyramidalized with the ribose positioned axially (Figure 2C,D) for puckering in either direction by the lowest free energy conformation

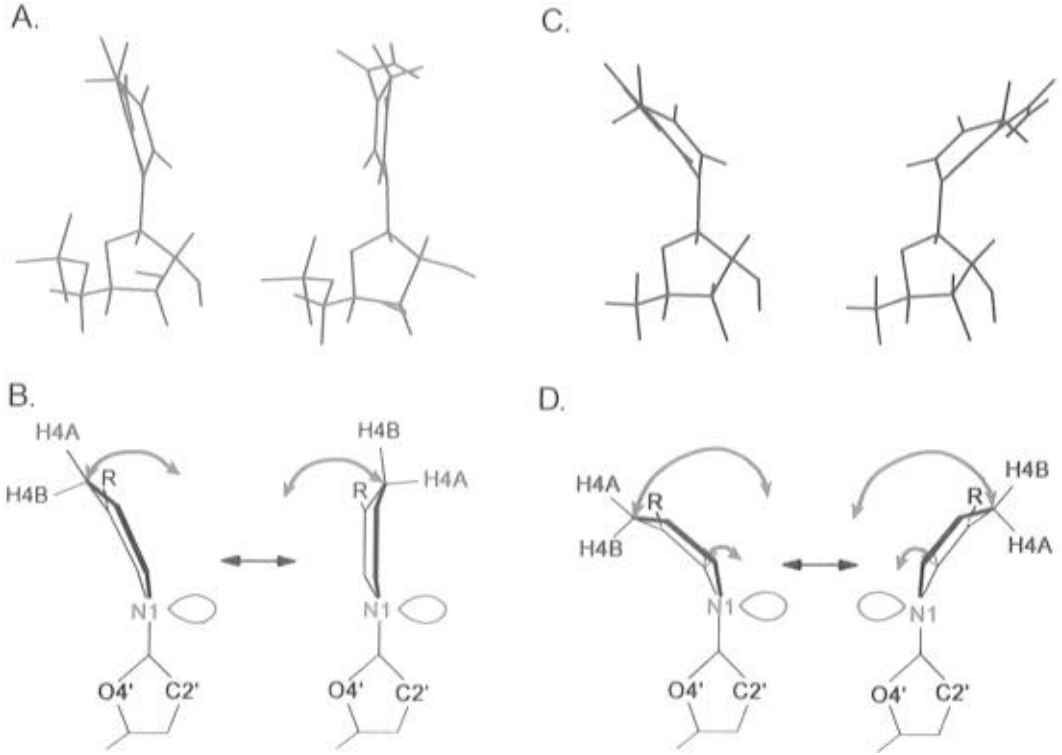

FIGURE 2: Conformations from thermal fluctuations in ring puckering of (A, B) *anti*-dihydronicotinamide for the LDH−NADH−pyruvate complex and (C, D) *in vacuo* NMSH, drawn (A, C) explicity and (B, D) schematically. Puckering in the direction of the A-side is shown on the right for each pair and puckering in the direction of the B-side, on the left. Of the four structures, only the structure on the right in A or B has the lone pair electrons pseudoaxial and resembles the transition state. The other three structures have the ribose pseudoaxial, unlike the transition state structure. Puckering toward C2′ yields the reactive ring conformation due to interactions with the nucleotide binding site. The lack of N1 inversion in going between the two puckering directions for bound NADH is the basis for the activation. The arrows in the schematic drawings (B, D) emphasize the difference in the displacements of atoms for the puckering motions. Conformations are time-averaged structures from studies to examine the free energy of boat-like puckering using thermodynamic perturbation methods of molecular dynamics simulations (Young & Post, 1993). The relative free energy differences calculated for these conformations is less than 1 kcal/mol, and the structures are puckered with N1 and C4 ±20° out of plane. The thermodynamic perturbation is along a reaction coordinate for boat-like conformations of the dihydronicotinamide ring defined in terms of only the six ring atoms. All other degrees of freedom (*e.g.*, N1 configuration and glycosidic torsion angle) were used in the generation of the ensemble of conformations for the partition function.

(Young & Post, 1993). Thus, transitions between the two pucker directions occur with inversion of N1 to give boat-like structures with either H4A or H4B in a pseudoaxial position but without simultaneously localizing the lone pair electron of N1 in a pseudoaxial position. In contrast, transitions in the enzyme active site between the two pucker directions do *not* involve N1 inversion (Figure 2A,B) due to the spatial confinement in the dinucleotide binding site. N1 pyramidalization remains constant for enzyme-bound NADH. Consequently, for A-side puckering (right-hand side in Figure 2A,B) the lone pair electrons of N1 are pseudoaxial simultaneously with H4A, as assumed for the transition state (Figure 1B). Because N1 does not invert between the two directions of puckering, thermal fluctuations lead to the reactive conformation in a stereospecific manner. For B-side puckering in LDH, the ribose is pseudoaxial (left-hand side in Figure 2A,B), as found for *in vacuo* NMSH, and unlike the transition state for hydride transfer.

This change in the puckering motion reflects the spatial restriction of N1 and atoms bonded to N1. Inversion of N1 in going between the two puckering directions requires the movement of atoms bonded to N1 and a large, concomitant displacement of the base, as illustrated by arrows in Figure 2D. Binding to LDH excludes fluctuations of such large amplitude. Nevertheless, the full range of boat-like puckering is accommodated within the dinucleotide binding site (Young and Post, manuscript in preparation) because of the second mechanism for puckering in which transitions between A-side and B-side puckering occur without inversion of N1 (Figure 2B). This type of motion allows ring puckering to the same extent, yet the displacement of any ring atom is small over the full range of puckering. The contrast in the volume required for puckering by these two mechanisms is well illustrated in Figure 3.

The architecture of the nucleotide binding site (the Rossmann fold) orients the base relative to the ribose such that N1 is pyramidalized in the direction shown in Figure 2A,B (as opposed to the opposite pyramidalization of N1 for *in vacuo* NMSH, right-hand side of Figure 2C,D). Contacts between conserved residues of the nucleotide binding fold determine the positions of C2 and C6 in NADH and maintain the N1 configuration that gives a reactive ring conformation for puckering toward the ribose C2′.

The enzyme-based origin for the correlation between the glycosidic torsion angle and stereospecificity of hydride transfer now can be readily understood from this puckering motion revealed for LDH. Binding of NADH positions the nicotinamide base relative to the ribose as shown in Figure 2A,B, and contacts between the protein and nicotinamide atoms N1, C2, and C6 determine the direction of N1 pyramidalization upon ring puckering. Consequently, puckering in the nucleotide binding site leads to a reactive

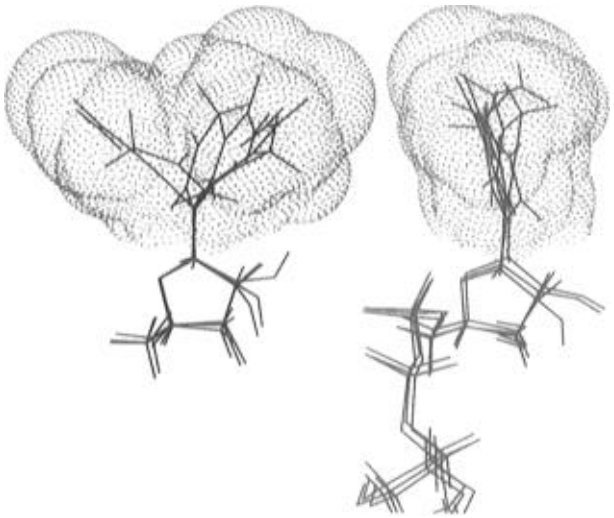

FIGURE 3: Comparison of the volume for ring puckering between puckering *in vacuo* and in the nucleotide binding site. Connolly surfaces enclose the base moieties corresponding to the full range of puckered conformations for *in vacuo* NMSH (blue) superimposed by a least-squares fit of the ribose atoms and for NADH in the complex LDH−NADH−pyruvate (red) superimposed by a least-squares fit of the LDH main chain.

conformation of the ring only when puckering is toward the ribose C2′. NADH binding in the *syn* orientation interchanges C2 and C6 positions relative to what is illustrated in Figure 2D. This *syn* orientation is shown in Figure 4A. By the same puckering mechanism found for *anti*-NADH, puckering toward C2′ with *syn*-NADH would place H4B in a pseudoaxial position in concert with a pseudoaxial position of the N1 lone pair electrons. Thus, the catalytic advantage from directed fluctuations promotes transfer of H4B in the case of binding *syn*-NADH.

This proposal for the enzyme-based origin of the correlation between glycosidic angle and stereospecificity depends on the orientation of the base relative to the ribose of the nicotinamide moiety being similar for A-stereospecific and B-stereospecific enzymes, except for the carboxamide moiety. This orientation is indeed found in the crystallographic structure of other A-stereospecific enzymes and the B-stereospecific enzyme glyceraldehyde-3-phosphate dehydrogenase, G3PDH (Skarzynski *et al*., 1987). The latter case is shown in Figure 4B with *syn*-NAD^+ from the crystallographic structure of the G3PDH complex superpositioned with the reactive conformation of *anti*-NADH with respect to the ribose atoms. There is a close fit of the base planes. Why the nucleotide binding domain has evolved such that puckering toward C2′, and not toward O4, yields the reactive ring conformation can be understood from the chemistry of hydride transfer of dihydronicotinamide (Wu & Houk, 1991).

In this report we have described a previously unrecognized property of enzymes whereby binding alters the distribution of conformations about a free energy minimum in a manner that promotes the reaction and explains the structural basis underlying the correlation between stereospecificity and glycosidic bond orientation; binding eliminates nonreactive conformations otherwise populated by thermal fluctuations about the minimum free energy conformation of free NADH to enhance conformations thought to be the reactive form of the dihydronicotinamide ring. Even though the conformation at the free energy minimum remains planar, similar to that of free NADH, we suggest that the active site geometry

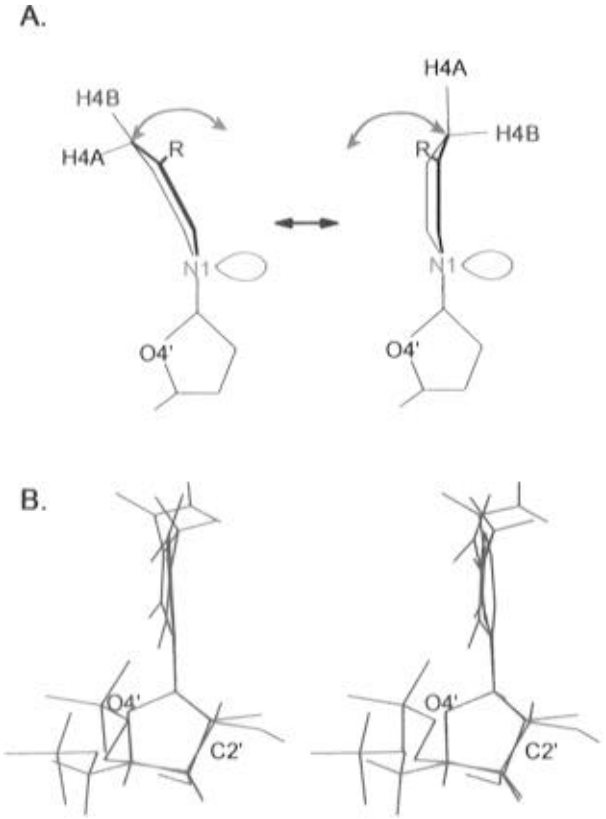

FIGURE 4: Predicted puckering motion for *syn*-dihydronicotinamide mononucleoside showing the structural basis for enhancing transfer of H4B in the site of a B-stereospecific enzyme. The puckering mechanism suggested by free energy simulations indicates that the reactive N1 configuration occurs with puckering fluctuations toward C2′ but not toward O4′. (A) For *syn*-NADH, puckering toward C2′ places H4B pseudoaxial in concert with the N1 lone pair electrons. This figure is analogous to Figure 2B, illustrating puckering of *anti*-NADH bound to A-stereospecific LDH. (B) The crystallographic coordinates (green) for the nicotinamide mononucleotide moiety of *syn*-NAD^+ complexed with G3PDH (Skarzynski *et al*., 1987), a B-stereospecific enzyme, are superimposed with *anti*-NADH for A-side puckering from the molecular dynamics simulation of LDH−NADH−pyruvate (red). The high degree of similarity in the orientation of the bases lends strength to the prediction that puckering toward C2′ activates the ring for hydride transfer, providing an enzyme-based origin for the correlation between stereospecificity of hydride transfer and glycosidic bond conformation.

confers a catalytic advantage not apparent from the equilibrium-averaged, ground state structure of the enzymatic complex. The enzyme exploits the loss of entropy due to association, beyond selectively binding one rotamer of a flexible cofactor, by using the restriction on thermally accessible NADH conformations to funnel the reactant toward the transition state. Selection of the reactive ring conformation with appropriate N1 pyramidalization among the thermal fluctuation conformations implies a more favorable entropy of activation in the enzyme-substrate complex relative to the uncatalyzed reaction (Warshel, 1984). As such, the enzyme has an entropic role with respect to activation of the dihydronicotinamide ring, in contrast to an enthalpic one of stabilizing an energetically unfavorable puckered conformation on average. The contribution to the enzymatic rate enhancement from such entropic guidance may be estimated following transition state theory (Espenson, 1981); the ratio of the enzyme-catalyzed reaction rate to the uncatalyzed rate, k_{enz}/k_{uncat}, is related to a difference in the activation entropies, $\Delta S^{\ddagger}$. If the entropy of the ring in the

transition state is similar for the enzyme-catalyzed and uncatalyzed reactions, then the difference in activation entropies reduces to a difference in the ground state entropy associated with the distribution of puckering conformations for NADH bound to LDH, S_B, compared to that for free NADH, S_F: $k_{enz}/k_{uncat} \propto \exp[(\Delta S^{\ddagger}_B - \Delta S^{\ddagger}_F)/R] \propto \exp[(S_B - S_F)/R]$. The difference $(S_B - S_F)$ may be a small contribution to the total rate enhancement. Nevertheless, the modulation of cofactor conformational fluctuations described here demonstrates a new means by which binding interactions influence the reaction coordinate and provides insight into stereospecificity. We hope that this work generates interest to determine whether such guidance of substrate or cofactor fluctuations toward transition state like geometries occurs in other enzymes.

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