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Conformational equilibrium of dihydronicotin amide in LDH-NADH

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Abstract

The dihydronicotinamide ring pucker of NADH bound to LDH is examined by exchange-transferred nuclear Overhauser nmr and by free energy molecular dynamics simulations. The influence of the enzyme-cofactor interactions on the conformational equilibrium of ring pucker was investigated in order to assess the potential role of steric strain in catalysis and stereospecificity of hydride transfer. Results presented here show that binding produces no constraint on the ring flexibility, therefore steric strain of the ring is not important. However, a second conformational feature - the configuration of the ring nitrogen - is perturbed by the enzyme and this effect may have consequences in catalysis.

INTRODUCTION

A small molecule ligand free in solution often has a large number of accessible conformational states which are distinguished primarily by rotation about dihedral torsion angles. Binding in the active site of an enzyme clearly must limit rotational freedom about torsion angles, however, some effort is needed to determine the exact nature of the restriction. It is of interest to make such an effort in order to evaluate what influence such restrictions might have on enzymic catalysis. Proper orientation of catalytic groups and steric strain have been described as mechanisms by which enzymes greatly enhance reaction rates (ref. 1). Orientation refers to proper alignment of groups for efficient catalysis and can be considered as selecting a particular rotamer for torsion angles of a sidechain or of the substrate. Steric strain, on the other hand, implies the binding of a high-energy conformation, such as a distorted ring conformation, by stabilization through intermolecular interactions.

One instance where conformation is thought to play an important role in reactivity is hydride transfer involving the nicotinamide ring of the enzymic cofactor nicotinamide-adenine dinucleotide (NADH). Hydride transfer is thought to occur from a puckered ring conformation whereby the transferring hydride is positioned axially to achieve the appropriate overlap of orbitals. As such, the direction of pucker corresponds to which proton is transferred. NADH is utilized in more enzymic reactions than any other cofactor (ref. 2), and the stereospecificity of reduction/oxidation of NADH is even thought to be a factor in the evolution of dehydrogenases (ref. 3).

In this article we discuss the interaction of NADH with lactate dehydrogenase (LDH). LDH is an $\alpha 4$ tetramer with each chain having 329 residues and catalyzes the reduction of pyruvate to lactate. The hydride transfer of this reaction is stereospecific for the A-side proton, H4A, of the dihydronicotinamide ring of NADH. The stereospecificity is greater than 10^7, the limit of the experimental measurement (ref. 4).

While a number of earlier studies have examined the energetics, transition states, and crystal and solution structure of isolated NADH, in this chapter we discuss the conformational equilibrium *in the enzyme active site.* That is, how does the surrounding protein environment alter the relative energy of the conformational states of the ring. Does the enzyme stabilize the pucker state appropriate for the stereospecificity of hydride transfer? Is the opposite pucker prevented by steric contact with protein residues?

Our study combines experimental nmr and computational molecular dynamics. Exchange-transferred nuclear Overhauser effects (ET-NOE) provide information on the bound state structure of small molecule ligands complexed to high molecular weight molecules. As this methodology can be applied to a number of important systems including drug-protein complexes, enzyme-substrate complexes, DNA binding ligands, and membrane binding ligands, part of our efforts have been to develop methods to obtain the most accurate 3-dimensional structures from ET-NOESY data (Schneider and Post, work in progress). Molecular dynamics studies expand upon the structural information obtained from ET-NOESY. Nmr experiments provide a single picture of the time-averaged conformational state, but a more detailed examination of the actual free energy surface can be obtained from thermodynamic simulations using conformationally constrained dynamics calculations. Information about the variation in conformations, or conformational flexibility, can thus be gained.

Hydride transfer of dihydronicotinamide

The transition state structure of dihydronicotinamide is thought to be a puckered conformation of the ring (ref. 5) in which the configuration of N1 has an axially positioned lone pair as shown in Fig. 1B. The lowest energy conformation is a planar ring and this conformation is that observed in the crystal state (ref. 6). However, the ring is easily deformable to a boat-like puckered conformation as found by nmr for the solution structure of NADH (ref. 7). To examine the possibility of stabilization of the puckered conformation of the ring by binding interactions in an enzymic active site, the puckered state was examined by nmr and computational studies for the LDH-NADH binary complex.

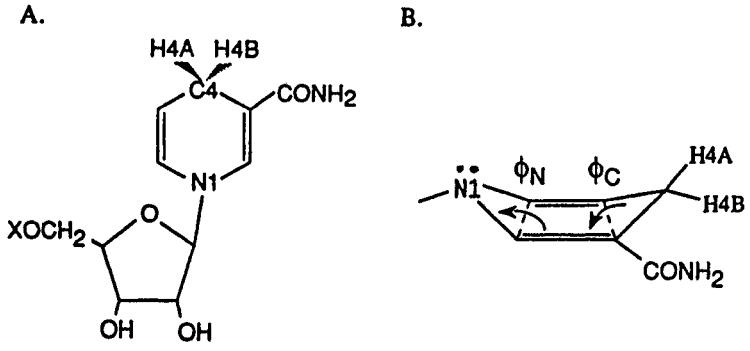

Fig. 1. A. Structure of NMSH (x = H) or NADH (x = adenine ribosyl pyrophosphate).
B. Reactive form of dihydronicotinamide for transfer of H4A.

NMR to determine bound state ring structure

It is possible to determine the conformation of the ring by measuring two interproton distances: H5--H4A and H5--H4B. For a planar ring, these distances are equal, and become unequal by an increasing amount as the ring puckers to greater degrees (Fig. 2). These two distances were measured by exploiting the exchange-transferred nuclear Overhauser effect (ET-NOE).

Nmr Structure Determination In Exchange Systems

Three-dimensional structure determination by nmr is based on the nuclear Overhauser effect (NOE). The NOE, measured from the change in the intensity of the off-diagonal peaks of a NOESY spectrum, is the result of dipolar interactions between two nuclei leading to cross-relaxation. The efficiency of the cross-relaxation is a function of the proton-proton separation (r^{-6}) and of the rotational correlation time (τ).

$$\sigma ij = F(r^{-6}ij, \tau)$$

Both the time-development of the NOE and the steady-state NOE are relevant to the work presented here.

The distance between two protons is measured from the time-development of the NOE. The rate at which the intensity of an off-diagonal cross-peak builds up as a function of the NOESY mixing time depends on the distance between the two protons i and j. In the limit of short mixing times, it is generally assumed that the intensity increases linearly with the cross-relaxation rate σ_{ij}, and therefore as r_{ij}^{-6}.

Not all molecules exhibit a strong NOE signal, as most readily demonstrated by considering the steady-state NOE value. The steady-state NOE of a two-spin system, corresponds to the maximum change in the relative intensity of one proton when a second proton is continuously irradiated. The steady-state NOE depends only on the correlation time τ. (In a NOESY spectrum, the relative intensity of the off-diagonal peaks will be less than the steady-state value.) For small molecular-weight molecules with short correlation times the NOE is positive, while for high molecular-weight molecules with long correlation times the NOE is negative. At intermediate correlation times, the NOE is near zero. That is, for certain size molecules the NOE is negligible regardless of the distance between two protons. The condition for the steady-state NOE equal to zero is $\tau = 1.12/\omega$, where ω is the spectrometer field strength. For 500 MHz, this value is $\tau = 0.3$ ns, the correlation time for small molecules approximately hundreds of daltons molecular weight.

Exchange-transferred NOESY (ET-NOESY) differs from directly measured NOESY by averaging of the system undergoing exchange. In an exchange system, a small molecule ligand, L, binds and dissociates from a large molecule protein, M.

$$M + L \rightleftharpoons M\text{-}L$$

As a result of the exchange process, nmr observables (e.g. chemical shift, linewidths, relaxation rates) are averaged according to the relative rate between exchange and the relevant nmr processes. For ET-NOE, the relevant processes are the binding rates, the self-relaxation rate ρ_i and the cross-relaxation rate σ_{ij}. For this work, the limit of fast exchange is satisfied since the binding rates for NADH to LDH are greater than 200 s^{-1}.

Because the fast-exchange limit applies, the interproton distance can be obtained from the initial slope of the ET-NOE time-development curve. For two protons i and j,

$$\text{slope} = x_b\sigma^b_{ij} + x_f\sigma^f_{ij}$$

Where x_b and x_f *are the mole fractions, and* σ^b_{ij} and σ^f_{ij} are the cross-relaxation rates of the bound and free ligand, respectively. As noted above, for molecules the size of NADH σ^f_{ij} is nearly zero. This condition was verified by measuring the NOE in the absence of enzyme.

Ring Structure Determined from ET-NOESY

The planarity of the dihydronicotinamide ring of NADH is defined from the two distances H5-H4A and H5-H4B (Fig. 2). In the case of a planar ring the distances are equal. For a boat-like puckered ring the distances are unequal with the direction of pucker defined according to the relative distances. Distances for these two pairs were obtained from the initial slope of the time-development of the ET-NOE. As shown in Fig. 3, the ET-NOE intensities as a function of mixing time are nearly equal for both proton pairs. Thus the average conformation of the ring in the active site of LDH is planar.

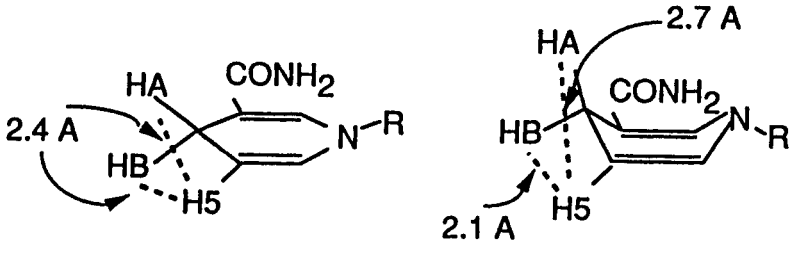

Fig. 2. Interproton distances measured by ET-NOESY to distinguish planar and puckered ring conformations.

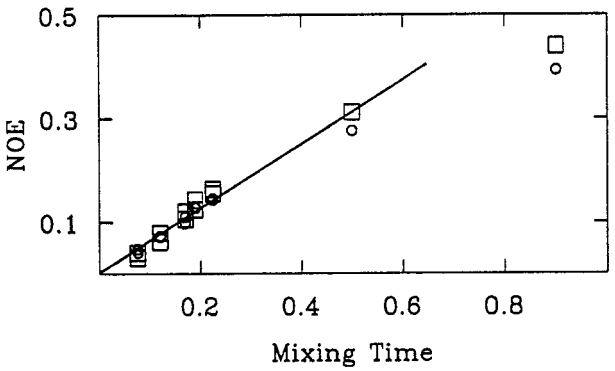

Fig. 3. ET-NOE build-up curve of H4A (o) and H4B (□) when H5 is irradiated. The sample contained 5 mM NADD (deuterated cofactor at either the A-side or B-side position of C4), and 0.5 mM LDH. Spectra were measured with a Varian VXR500 spectrometer at 8C.

Free energy profile for ring pucker

A deeper understanding of the conformational equilibrium for ring pucker was obtained from free energy molecular dynamics simulation studies. While nmr provides information on the time-averaged conformational state of the ring, the relative free energy for the range of puckered conformations can be calculated by simulation methods.

Simulation Methodology

Free energy differences between two closely related states can be computed by using thermodynamic simulation techniques. The theoretical foundation of this methodology has been known for some time, and the rapid growth in computational ower has allowed its recent application through molecular dynamics to a large number of biological systems (ref. 8). To determine conformational equilibria by thermodynamic perturbation methods, the reaction coordinate is specified in terms of internal coordinates, instead of chemical identity of atoms as often used in thermodynamic calculations to study, for example, binding free energies or effects of amino acid mutations.

The reaction coordinate, S, for ring puckering of dihydronicotinamide is defined as a function of two dihedral angles: ϕC4-C5-C3-C6 ($=\phi C$) and ϕC5-C6-C2-N1 ($=\phi N$) (Fig. 1B). S is a linear combination of these angles determined from the ϕC versus ϕN potential energy surface obtained by energy minimization with harmonic constraints on the dihedral angles (ref. 9). The reaction coordinate includes planar and boat configurations with puckering in both directions and is defined as

$$S(\phi C, \phi N) = -1.667 \times 10^{-2}\phi C + 1.944 \times 10^{-2}\phi N \qquad \text{Eq. 1}$$

Let S = 0 correspond to (140,120) and S = 1 correspond to (220,240). The planar ring then occurs for a value of S = 0.5.

To calculate the free energy difference between two pucker states given by different S values, we use the exponential form of the perturbation simulation method (ref. 8). For states i and j,

$$\Delta A = A_j - A_i = -kT\ln \langle e^{-\beta}(E_j - E_i) \rangle_i \qquad \text{Eq. 2}$$

k is Boltzmann's constant, T is the absolute temperature, β = 1/kT, and E_i is the potential energy of the i^{th} conformational state. The expression $\langle \rangle_i$ is the statistical mechanical ensemble average evaluated from the simulation representative of state i.

To calculate the free energy profile for ring pucker, the full range of pucker was divided into small conformational changes and the individual values of ΔA combined to construct the complete profile. The dihedral-angle internal coordinates given by $S(\phi C, \phi N)$ were constrained during the dynamics by using holonomic constraints according to Tobias and Brooks (ref. 10). The trajectory structures were 'perturbed' by using values of δS ranging from 4^o to 12^o to determine ΔA (eqn. 2) for small increments in ring pucker (ref. 9). The free energy profile for the full range of pucker was therefore calculated with perturbations obtained from seven simulations referenced to a particular $S(\phi C, \phi N)$.

Ring-Pucker Free Energy for Isolated and Bound Dihydronicotinamide

The free energy for boat-like puckering of the six-membered ring was calculated for the isolated molecule dihydronicotinamide mononucleoside (NMSH) (Fig. 4) and NADH bound to LDH. Calculations with the isolated NMSH were important not only for comparison purposes but for establishing the best procedure for applying a conformationally constrained perturbation approach.

From the isolated NMSH calculations, it was found that a rigid conformational perturbation can lead to an error in ΔA when δS is too large, even though $\Delta A \sim 0$, (ref. 9). The perturbation applied with the holonomic constraint is a rigid displacement along the reaction coordinate which can deviate from the minimum energy path and hence lead to an erroneously large value for ΔA. As a consequence of this finding, δS was reduced for calculations involving the planar ring and the error in ΔA was insignificant.

ΔA values for NMSH were calculated at each S*i* from five simulations with different initial velocities, and the averages used to construct the profile shown in Fig. 4. The free energy curve for NMSH has a broad minimum at S = 0.5 corresponding to the planar ring and increases rapidly only for pucker angles beyond approximately $\phi C = 180 \pm 20^{\circ}$ and $\phi N = 180 \pm 30^{\circ}$.

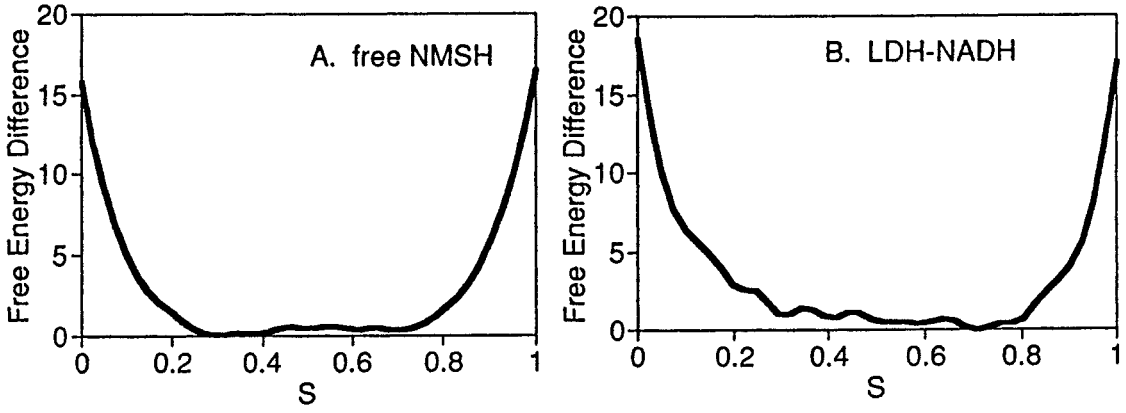

Fig. 4. Free energy profiles calculated from constrained molecular dynamics for the ring pucker coordinate S. The planar ring has S = 0.5, and the puckered ring has S <0.5 or S >0.5 for the two directions of pucker. A. Free NMSH calculation. B. Calculation for NADH bound to LDH.

The ring pucker free energy profile (Fig. 5B) was calculated for the binary complex LDH-NADH following the same procedure as that for the isolated NMSH; only the two dihedral angles ϕC and ϕN were constrained according to S (eqn.1) while sampling all other conformational degrees of freedom. Comparison of the profiles shown in Fig. 5A and 5B finds that the free energy of puckering is nearly identical for bound NADH and isolated NMSH. That is, in spite of the surrounding protein environment, the ring flexibility is unaffected. Neither is the degree of puckering reduced, nor is there a preference for puckering in one direction.

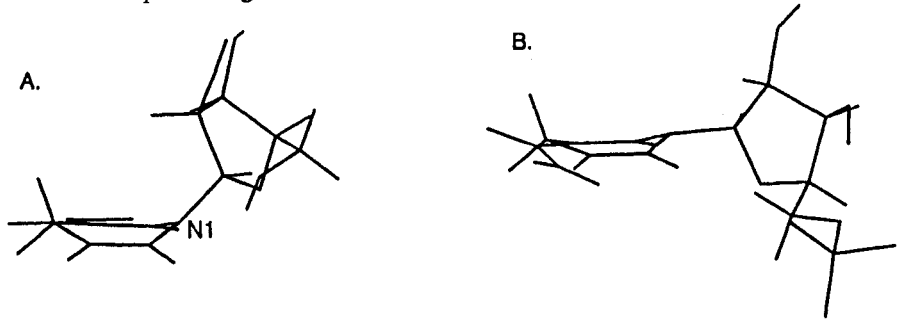

Fig. 5. Structures from the simulations of A.) isolated NMSH and B.) LDH-NADH constrined to the puckered conformation 5 = 0.3. The structures differ in the configuration of N1.

Rather than stabilizing the ring in a puckered conformation, the simulation suggests the catalytic power of LDH derives in part from affecting an alternative conformational factor – the configuration of the ring N1. Molecular dynamics sampling with ϕC and ϕN constrained to give a ring pucker in the direction corresponding to hydride transfer of H4A (i.e. S = 0.7) lead to the average structures for isolated NMSH and bound NADH shown in Fig. 5. The isolated NMSH ring has the lone pair positioned equatorially, the minimum energy position. In contrast, the lone pair is positioned axially for the *bound* NADH ring, the configuration most suited for hydride transfer. Furthermore, when the ring is constrained in the opposite direction of pucker (i.e. S = 0.3) the lone pair of N1 is equatorial for both isolated and bound dihydronicotinamide. Thus interactions in the active site of LDH promote inversion of the ring N1 so that both H4A and the lone pair are well positioned for stereospecific hydride transfer.

In addition, the distribution of crystallographic waters observed in the active site of LDH was noted. Although the nicotinamide moiety of NADH is completely buried, the A-side of the ring is hydrated. This polar environment for the side of the ring where transfer occurs is consistent with the redistribution of charges which accompanies bond breaking and bond making. The B-side of the ring binds in a nonpolar environment. A small number of the active-site waters move during the transition from one pucker direction to the other.

CONCLUSIONS

The conformation of the dihydronicotinamide ring of NADH bound to LDH has been examined by using ET-NOESY experiments to determine if the ring is planar or in a boat-like pucker. If a puckered ring were stabilized by intermolecular interactions, then steric strain, whereby a distorted, high-energy ring conformation is maintained, would play a role in catalysis. Selective enhancement of one direction of pucker would give some explanation for the enormous stereospecificity of this enzyme as well.

However, the nmr results show the ring to be planar in the LDH-NADH complex. The more detailed picture provided by thermodynamic simulation techniques, in agreement with the nmr results, gives a broad free energy curve with a minimum for the planar ring, and indicates that confinment by enzymic groups in the active site does not alter the free energy of puckering relative to the unbound ring. Enzymes do *not* restrict the ring conformational states of substrates. Although a large volume is swept out for the transition between the two directions of ring puckering for unbound NMSH, only a small volume is occupied by bound NADH for the entire S coordinate as a result of small changes in angles and torsion angles. Flexibility of the ring is not hindered.

It appears that the catalytic advantage is the result of a concerted orientation of groups appropriate for efficient catalysis. Rather than constraining the ring in a high energy state, the active site binds NADH such that when the ring is puckered in the correct direction for the stereospecific hydride transfer, both H4A and the N1 lone pair are axially positioned. The concerted orientation of the NADH groups and asymmetric polarity of the active-site environment explain in qualitative terms the stereospecificity of the reaction.

REFERENCES

1. Walsh, C., In *Enzymatic Reaction Mechanisms,* Freeman, San Francisco, ch. 2. (1979).
2. You, K., *CRC Crit. Rev. Biochem.* **17**: 313-451 (1985).
3. Benner, S. A., *Experientia* **38**: 633-637 (1983).
4. LaReau, R. D. and Anderson, V. E., *J. Biol. Chem.* **264**: 15338-15343 (1989).
5. Wu, Y-D. and Houk, K.N., *J. Am. Chem. Soc.* **109**: 2226-2227 (1987).
6. Glasfeld, A., Zbinden, P., Dobler, M., Benner, S. A. and Dunitz, J. D., *J. Am. Chem. Soc.* **110**: 5152-5157 (1988).
7. Oppenheimer, N. J., In *Pyridine Nucleotide Coenzymes, Chemical, Biochemical and Medical Aspects,* Wiley, New York (1987).
8. Mezei, M. and Beveridge, D. L., In *Computer Simulation of Chemical and Biomolecular Systems,* New York Acad. Sci., New York (1986).
9. Young, L. and Post, C. B., *J. Am. Chem. Soc.,* **115**: 1964-1970 (1993).
10. Tobias, D. J. and Brooks, C. L., *J. Chem. Phys.* **89**: 5515-5527 (1988)