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An Inquiry into the Source of Stereospecificity of Lactate Dehydrogenase Using Substrate Analogues and Molecular Modeling†

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ABSTRACT: Lactate dehydrogenase catalyzes the stereospecific hydride transfer to and from the *re* face of the nicotinamide coenzyme. The demonstrated probability of transfer to the *si* face of less than 2×10^{-8} indicates that the free energy of any diastereotopic transition state leading to a *si* transfer must be over 10 kcal/mol greater than the free energy for transfer to or from the *re* face. The general notion of closed, desolvated active sites suggests the a priori hypothesis that steric hindrance prevents the nicotinamide ring from assuming a conformation that would lead to transfer of the *pro-S* hydrogen. In this paper we report that the probability of transfer of the *pro-S* proton is less than 9×10^{-7} with 3-pyridinealdehyde adenine dinucleotide as coenzyme and less than 4×10^{-7} during the lactate dehydrogenase catalyzed disproportionation of glyoxylate. Examination of the crystal structure of lactate dehydrogenase further suggests that steric exclusion does not enforce the extreme stereospecificity of the reaction. An electrostatic interaction with the macrodipole associated with the α2F helix is suggested as a potential molecular source of the stereospecificity.

The pyridine nucleotide dependent dehydrogenases catalyze the reversible hydride transfer to and from the C-4 carbon of the nicotinamide moiety of $NAD(P)^+$. It has been well established that this reaction is stereospecific, such that a given dehydrogenase transfers either the *pro*-4*R* (A-side) or *pro*-4*S* (B-side) hydrogen of the coenzyme (Fisher et al., 1953; Loewus et al., 1953; Jarabak & Talalay, 1960; Cornforth & Ryback, 1962; Arnold et al., 1976; You, 1985). In our original experiments, the reaction

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$$[4\text{-}^3H]NAD^+ + \text{lactate} \rightleftarrows (4S)\text{-}[4\text{-}^3H]NADH + \text{pyruvate}$$

under conditions of dynamic equilibrium as catalyzed by LDH[1] was monitored. If this catalyzed reaction were 100% stereospecific (always *pro*-4*R*), the tritium label would remain on the dinucleotide and never be transferred to the lactate. If however, the enzyme were to catalyze a stereochemically improbable *pro*-4*S* hydride transfer, i.e., either to or from the *si* face of the nicotinamide, then the tritium label would be ultimately transferred to pyruvate to form [2-^{3}H]lactate. Because the sample of [4-^{3}H]NAD$^+$ in these experiments does not turn over just once, but ca. 10^5 times, there is a large "amplification" of an otherwise undetectable rate of nonstereospecific hydride transfer. By knowing the specific activity of the initial [4-^{3}H]NAD$^+$ sample (total [^{3}H]NAD^+_i cpm), the total number of hydride transfers (N), and the amount and specific activity of the final isolated [2-^{3}H]lactate (total [^{3}H]lactate$_f$ cpm) after the reaction is quenched, one can determine the ratio of nonstereospecific hydride transfers to the absolute number of hydride transfers, i.e., the probability (P) that any individual hydride transfer is *pro*-4*S*, by

$$P = (\text{total } [^3H]\text{lactate}_f \text{ cpm})/(\text{total } [^3H]NAD^+_i \text{ cpm})(N) \quad (1)$$

By this method we have been able to increase the sensitivity of these stereospecificity determinations by over 6 orders of magnitude relative to the classical "single-turnover" experiments (Anderson & LaReau, 1988; LaReau & Anderson, 1989).

For the system of porcine heart lactate dehydrogenase which we have studied, the probability of the nonstereospecific (*pro*-4*S*) hydride transfer has been established to an *upper* limit of 2.4×10^{-8}. For every order of magnitude of increase in stereospecificity there is a 1.36 kcal/mol difference at 25 °C in the relative stabilization energies of the two diastereotopic transition states that lead to transfer of either the *pro*-4*R* or the *pro*-4*S* hydrogens. This indicates that the *pro*-4*R* hydride transfer is favored over the *pro*-4*S* hydride transfer by over 10 kcal/mol. This difference in the energy of the diastereotopic transition states leading to hydride transfer *must* be the result of specific interactions of the 3-substituent of the pyridine ring, because without the 3-substituent, the *re* and *si* faces of the ring would be indistinguishable by a 180° rotation about the glycosidic bond. In the experiments described here, substrate analogues and molecular modeling are used to support the hypothesis that an attractive interaction between the carbonyl of the C-3 substituent and the enzyme is responsible for the extreme stereospecificity of the LDH reaction and is essential for catalysis.

Materials and Methods

Stereospecificity Determinations Using Substrate Analogues. The protocols and calculations for the stereospecificity determinations using NAD$^+$ and lactate (the natural substrates) are described previously (LaReau & Anderson, 1989). The protocols for the stereospecificity determinations using 3-pyridinealdehyde adenine dinucleotide (3-pyrAl-AD$^+$) in place of NAD$^+$, and those using glyoxylate in place of lactate, are essentially identical. Changes are described in detail below.

Synthesis of [4-^{3}H]-3-PyrAl-AD$^+$. A solution of 30 mM sodium 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid (TAPS) buffer, pH 7.8, containing 12 mM magnesium acetate, 3.2 μM [1-^{3}H]glucose (30 Ci/mmol) Amersham, 300 μM ATP, and 3 units of hexokinase in a total volume of 800 μL catalyzed the formation of [1-^{3}H]glucose 6-phosphate. NAD$^+$ was then added to 10 μM with 2 units of glucose-6-phosphate dehydrogenase to form (4*S*)-[4-^{3}H]NADH. The 3-fold excess NAD$^+$ assures complete oxidation of the [1-^{3}H]glucose 6-phosphate. 3-pyrAl-AD$^+$ was then added to the solution to 10 μM and allowed to remain for 12 h, at which point oxaloacetate was added to 0.1 mM with 5 units of malate dehydrogenase to catalyze the oxidation of all NADH and 3-pyrAl-ADH to NAD$^+$ and 3-pyrAl-AD$^+$. The entire solution was titrated to pH 3 with formic acid and injected directly onto a 1- × 25-cm Econosil C-18 reverse-phase column (Alltech). 3-PyrAl-AD$^+$ eluted in a broad (3–5-mL) but well-defined peak which was collected, and the solvent was removed by centrifugal vacuum evaporation. The specific activity of the 3-pyrAl-AD$^+$ was approximately 500 mCi/mmol. To ensure that the isolation of 3-pyrAl-AD$^+$ excludes any contaminating NAD$^+$, a trace amount of [4-^{3}H]NAD$^+$ was added to a nonradioactive sample of 3-pyrAl-AD$^+$ and the mixture was immediately purified under the standard HPLC conditions. There were no detectable ^{3}H counts above background from the 3-pyrAl-AD$^+$ peak up to and including the 27–28-min fraction (NAD$^+$ elutes at 29–30 min). This ensures that the enzymatic turnover we observed resulted from 3-pyrAl-AD$^+$ activity and was not due to a trace amount of NAD$^+$ in the exchange solutions.

3-PyrAl-AD$^+$ Stereospecificity Determinations. The exchange solution and kinetics assay were identical to those described previously for the NAD$^+$ stereospecificity experiments. The only two changes were that [4-^{3}H]-3-pyrAl-AD$^+$ was used in place of [4-^{3}H]NAD$^+$, and the time delay for the ^{1}H-NMR kinetics acquisition program was increased from 5 to 10 min. The exchange rate, as determined by the [$^2H_3$]-pyruvate to [$^2H_3$]lactate flux monitored by ^{1}H-NMR, was 8–10 times slower relative to the NAD$^+$-supported rate for porcine heart LDH. The exchange solution was allowed to remain for 24 h, at which point the reaction was stopped and the [2-^{3}H]lactate isolated as crystalline Zn(lactate)$_2$·(H$_2$O)$_3$ as previously described in the NAD$^+$/lactate experiments (LaReau & Anderson, 1989).

Glyoxylate Stereospecificity Determinations. The procedure for the stereospecificity determinations using glyoxylate was altered slightly in order to accommodate the substrate analogue. Glyoxylate acts as both an oxidant and a reductant for the LDH reaction (Warren, 1970). The keto form oxidizes NADH to form NAD$^+$ and glycolate, while the hydrated form reduces NAD$^+$ to form NADH and oxalate. The enzymatic turnover was run in D$_2$O containing 100 mM phosphate buffer (pD 7.5), 200 mM glyoxylate, and 10 μM [4-^{3}H]NAD$^+$. Three hundred units of LDH resuspended in D$_2$O was added to the reaction solution, and ^{1}H-NMR was used to monitor the conversion of glyoxylate to glycolate (as determined by the appearance of the glycolate C-2 proton resonance at 3.6 ppm and the disappearance of the glyoxylate C-2 proton resonances at 5.2 ppm). It was unnecessary to determine the enzymatic turnover rate in these experiments. When 100 mM glyoxylate had been disproportionated (as monitored by NMR), the reaction was quenched by the addition of 1.0 g of glycolic acid and 2.0 mL of H$_2$O. The total number of hydride transfers (N) is then

$$N = [\text{glyoxylate}]/[[4\text{-}^3H]NAD^+] = 10^4 \quad (2)$$

[1] Abbreviations: DMV, *N*,*N*-dimethylvaline; LDH, lactate dehydrogenase; 3-pyrAl-AD$^+$(H), 3-pyridinealdehyde adenine dinucleotide (reduced); TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

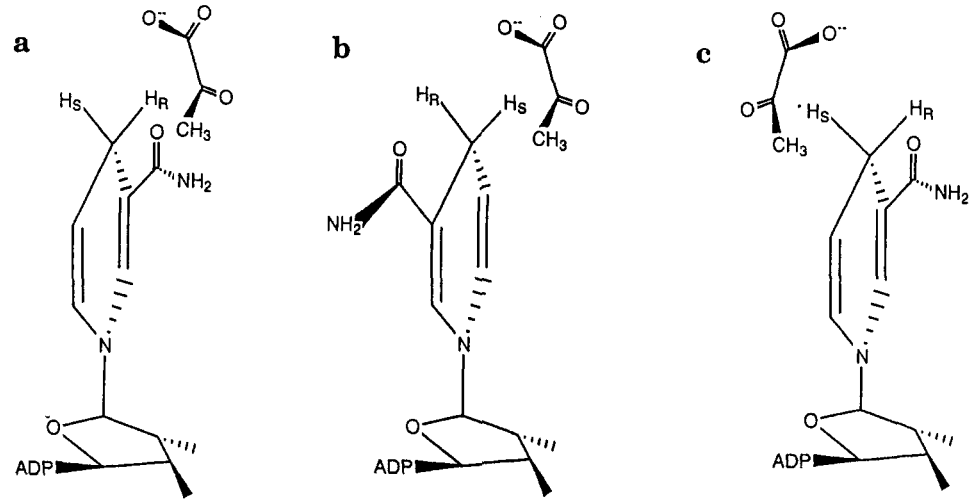


FIGURE 1: Relative spatial orientation of the reduced nicotinamide ring and pyruvate. (a) The crystallographically and stereochemically determined orientation. The nicotinamide ring is anti and will transfer the *pro-4R* hydrogen to form L-lactate. (b) With the nicotinamide ring bound in the syn conformation, transfer of the *pro-4S* hydrogen produces L-lactate. (c) With pyruvate bound on the *si* face of the nicotinamide ring in the anti configuration, transfer of the *pro-4S* hydrogen produces D-lactate.

The entire sample was passed through an activated charcoal column, and the glycolate was isolated as the Zn^{2+} salt and recrystallized from hot water to constant specific activity.

Relative Glyoxylate V/K Determinations. The relative rates of reduction of glyoxylate and pyruvate by NADH as catalyzed by LDH were determined by measuring the initial reaction rates under conditions of 30 mM Tris-HCl, pH 7.5, 0.24 mM NADH, and 5 mM pyruvate or glyoxylate. The initial rates of disappearance of NADH were monitored by the change in absorbance at 340 nm.

Separation of D-[*2-*^{3}*H*]*Lactate and* L-[*2-*^{3}*H*]*Lactate.* After four standard stereospecificity experiments were performed using the natural substrates (NAD^+ and lactate), D-lactate was added to 50 mM to the exchange solutions and the lactate was isolated under conditions to separate and quantify the relative amounts of the radioactive D- and L-isomers. For the first two experiments, the lactate was isolated after charcoal column filtration as previously described and subsequently reisolated by HPLC with the elution protocol described for NAD^+. Lactate eluted from the column under these conditions at 5–8 min, and the fractions containing lactate were pooled and dried. The lactate samples were then oxidized in a solution containing 100 mM Tris buffer (pH 7.5), 20 mM NAD^+, 100 mM semicarbazide, and 100 units of either D- or L-lactate-specific LDH. This catalyzed the transfer of the C-2 proton (or tritium) of lactate to the *re* face of NAD^+ to form NADH. The *pro-4S* proton of NADH was subsequently removed by the addition of α-ketoglutarate and ammonium sulfate to 50 mM with 10 units of glutamate dehydrogenase. The solutions were quenched to pH 3 with formic acid, and the NAD^+ was isolated and counted for ^{3}H content.

In the other two experiments, D-lactate and L-lactate were separated chromatographically by dissolving the isolated and dried lactate samples (see above) in 5 mL of a 16 mM Cu(dimethylvaline)$_2^{2+}$ [Cu(DMV)$_2$] solution before injecting them on the Econosil reverse-phase column equilibrated with 5% MeOH and 95% H_2O (Benecke, 1984). The Cu(DMV)$_2$·D-lactate$_2$ complex elutes between 3 and 4 min, while the Cu(DMV)$_2$·L-lactate$_2$ complex elutes between 4 and 5 min. The two peaks were collected manually. While not completely base line resolved, we estimate that each enantiomer should not be contaminated by any more than 20% of the total of the other. The separated fractions containing the D- and the L-isomers were each counted for ^{3}H content.

Scintillation Counting. All ^{3}H samples were dissolved in 7 mL of Ecolume scintillant and counted with a Beckman LS 5000 scintillation counter.

Molecular Graphics. The crystal structures of pig heart LDH with (3*S*)-5-(3-carboxy-3-hydroxypropyl)-NAD^+ [5-(*S*)-lac-NAD^+] bound at the active site (5LDH; Grau et al., 1981), of dogfish muscle LDH with NADH and oxamate bound at the active site (1LDM; Abad-Zapatero et al., 1988), and of cytoplasmic malate dehydrogenase (4MDH; Birktoft et al., 1989) with NADH bound at the active site were obtained from the Brookhaven Protein Data Bank and manipulated with the QUANTA (Polygen) interface to CHARMm (Brooks et al., 1983) running on a personal IRIS graphical workstation.

Results and Discussion

Stereospecificity Determinations Using 3-PyrAl-AD$^+$. Lactate dehydrogenase displays a phenomenal fidelity in the stereospecificity of the reaction it catalyzes. Similar extreme stereospecificity has been demonstrated for yeast alcohol dehydrogenase (Benner, personal communication). It is of interest both to determine the mechanism for this induced stereospecificity and to ask the question whether such a high fidelity is intrinsically important in the catalytic mechanism, or whether it is simply enforced by the geometry of the active site. The simplest model which could account for the stereochemical discrimination displayed by LDH is one in which the nicotinamide moiety of the coenzyme binds into a sterically restricted active site pocket such that the carboxamide arm can be placed in only one conformation relative to the nicotinamide ribose ring (an anti conformation in this case). Presumably, if the nicotinamide ring were able to rotate 180° about the glycosidic bond and bind in a syn conformation instead, then the catalyzed hydride transfer would occur at the *si* face (B-side) rather than the *re* face (A-side) of the ring (Figure 1a,b). The only molecular change introduced by this rotation is the position of the substituent at C-3.

We repeated our original experiments using the NAD^+ analogue, 3-pyridinealdehyde adenine dinucleotide (3-pyrAl-AD$^+$), in which the carboxamide arm is replaced by an aldehyde group. This was done in order to determine whether interactions of the NH_2 moiety at the active site generate the observed stereospecificity. The only interaction of the entire carboxamide arm noted in the crystal structure is a potential

Table I

dinucleotide	cosubstrate	maximum probability of nonstereospecific transfer[a]	minimum relative diastereotopic stabilization energy difference (kcal/mol)[b]
NAD^+	lactate	$(2.4 \pm 1.7) \times 10^{-8}$	10.4
3-pyrAl-NAD^+	lactate	$(9.4 \pm 9.8) \times 10^{-7}$	8.2
NAD^+	glyoxylate	$(4.3 \pm 2.5) \times 10^{-7}$	8.7

[a] As calculated by eq 1, see text. The reported values and standard deviations represent the averages of five separate experiments for the NAD^+/lactate substrate pair and three separate experiments for both the 3-pyrAl-NAD^+/lactate and the NAD^+/glyoxylate substrate pairs. [b] $RT \ln (P)$.

hydrogen bond from the amide group to Ser-163 (Grau et al., 1981). The elimination of the amide removes the possibility for this hydrogen bond as a mode for anti stabilization, as well as reduces the potential for unfavorable steric interactions if the pyridine ring were to bind in the syn conformation.

The measured probability of transferring the *pro*-4*S* hydrogen in the 3-pyrAl-AD^+ reaction appears to have increased slightly relative to that of NAD^+ to 9.4×10^{-7} (Table I). Both of these determinations, however, are *upper* limits on the probability of nonstereospecific transfer and are limited by the number of turnovers. Consequently, the contribution of the amino functionality cannot be determined by difference, as the lower limit may be attributed to fewer turnovers of the nucleotide. What is important is that the extreme stereospecificity is maintained, indicating that interactions of the C-3 substituent (which now consists only of a carbonyl group) with the active site favor the transition state where the *pro*-4*R* hydrogen is transferred by over 8 kcal/mol.

Stereospecificity Determinations Using Glyoxylate. Another possible mode by which the syn conformation of NAD^+ could be prevented due to steric hindrance in the bound ternary complex comes from the cosubstrate lactate/pyruvate. Noting the required relative conformations of the bound substrates, Grau (1982) suggested that the stereochemistry of the reaction might be determined by steric hindrance between the methyl group of the lactate or pyruvate and the carboxamide arm of the nicotinamide ring if the coenzyme were bound in a syn conformation. Since the syn conformation of the nicotinamide ring would be prevented by the cosubstrate, the coenzyme must adopt an anti conformation in the ternary (but not necessarily the binary) complex, making the hydride transfer always *pro*-4*R*.

In order to test the contribution of the methyl group of the lactate and pyruvate to the stereospecificity of the reaction, we repeated the experiments using glyoxylate, which lacks the C-3 methyl group, as the cosubstrate. Glyoxylate both oxidizes NADH (forming glycolate) and as the hydrate reduces NAD^+ (forming oxalate) for the lactate dehydrogenase reaction (Warren, 1970). For this reason our experimental procedures were altered slightly from the experiments with lactate as the substrate (see Materials and Methods). The results of these experiments showed an increase in the maximum probability of nonstereospecific transfer by a factor of about 20 relative to that for the natural substrates (Table I). As with pyrAl-AD^+, this apparent increase may be due to the lower number of turnovers with the alternative substrate. So while this indicates that mutual exclusion of the carboxamide arm and the methyl group may be a source of some of the stereospecificity, there remains a diastereotopic stabilization energy of at least 8 kcal/mol that is independent of the cosubstrate methyl group. We recognize that the hydrate of glyoxylate will probably have a hydroxyl group in place of the methyl group during the reduction of NAD^+, but during the oxidation of NADH the keto form of glyoxylate, with a hydrogen atom substituted for the methyl group of pyruvate, is the presumptive substrate.

Table II: Quantitation of D- and L-[2-^{3}H]Lactate (cpm)

separation	D-lactate	L-lactate
Cu(dimethylvaline)$_2^{2+}$ HPLC[a]	5060	5640
enzymatic[b]	630	370

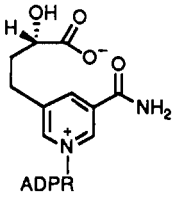
[a] D- and L-lactate separation done by HPLC of the Cu(DMV)-lactate complex (see Materials and Methods). [b] D- and L-lactate were distinguished with D- and L-lactate-specific LDHs.

Potential Diastereotopic Transition States. In the stereospecificity determinations using both the natural substrates and the substrate analogues, we were always able to detect at least a small amount of nonstereospecific transfer. This nonstereospecific transfer, if catalyzed by the enzyme, could be the result of one or more alternative transition-state structures. Two of these possibilities are especially worth noting. X-ray crystallography of both binary and ternary complexes of nucleotides with lactate dehydrogenase has indicated that the nicotinamide ring is bound in an anti conformation with C-2 of lactate or pyruvate located on the *re* face of the ring as shown in Figure 1a (Grau et al., 1981; Chandrasekhar et al., 1973). *pro*-4*S* hydride transfer between the ring and pyruvate or lactate requires either the nicotinamide ring to rotate about the glycosidic linkage as shown in Figure 1b or the cosubstrate unit to bind on the *si* face as shown in Figure 1c. If the *pro*-4*S* transfer is the result of the nicotinamide ring bound in a syn rather than an anti conformation, the hydride transfer to pyruvate will form L-[2-^{3}H]lactate. If the pyruvate is bound with the C-2 carbonyl bond still directed toward His-195, as in the crystallographic complex, hydride transfer from the *si* face of the nicotinamide ring will generate D-[2-^{3}H]lactate. Both enantiomers of lactate would be detected by our protocol.

In order to determine which of these two modes of hydride transfer may be the major source of nonstereospecificity (i.e., the source of tritium isolated as [^{3}H]lactate), carrier racemic lactate was added to an experimental run which used the natural substrates (lactate and [4-^{3}H]NAD^+). Subsequently, D-lactate was separated from L-lactate both enzymatically and by HPLC (Benecke, 1984) and the specific activity of each enantiomer was determined (Table II). The enzymic analyses using D- and L-specific lactate dehydrogenase indicate that [2-^{3}H]lactate is indeed the radioactive product because the radioactivity can be transferred back to the coenzyme. Results show that the specific activities for each of the two lactate enantiomers are nearly identical, indicating either that there are at least two modes of catalyzed nonstereospecific transfer which have similar energies of stabilization or that the tritium label we observe comes from a nonstereospecific background reaction. As the rate of enzymatic turnover is significantly slower for the analogues than for the natural substrates [rates for 3-pyrAl-AD^+ have been determined to be 3–10 times slower than for NAD^+ with LDH (Anderson, 1982) while glyoxylate rates are 25 times slower than those for pyruvate under our experimental conditions (see Materials and Methods)], a non-enzyme-catalyzed background reaction would contribute a larger percentage of nonstereospecificity to the otherwise stereospecific enzyme-catalyzed reactions. Controls with [4-^{3}H]NAD^+ and [4-^{3}H]NADH suggest the nonenzymatic reaction would generate less than 1% of the observed [2-^{3}H]lactate following correction for the amount of free nucleotide present (LaReau & Anderson, 1988), but we cannot rule out that there are other sites on the surface of LDH that lead to a small rate enhancement of a nonstereospecific hydride

transfer between [4-^{3}H]NADH and either pyruvate or [4-^{3}H]NAD$^+$. For this reason, we are not confident that we have truly identified *any pro*-4*S* transfer catalyzed at the active site of lactate dehydrogenase and that the probabilities of nonstereospecific transfer we have reported are strictly *upper limits*.

Molecular Modeling. If the active site residues of the enzyme were situated such that rotation about the glycosidic bond of the NAD$^+$ was restricted, then the stereospecificity would simply be a function of the coenzyme's inability to bind (either the ground state or the transition state) any other than the crystallographically observed anti conformation. Oppenheimer et al. (1986) attribute the stereospecificity of horse liver alcohol dehydrogenase to steric exclusion by Cys-175 and Gly-174. In 5-(*S*)-lac-NAD$^+$, the methylene bridge between the nicotinamide ring and lactate occupies the same relative position that the carboxamide arm would occupy following a 180° rotation about the glycosidic bond. This analogue, as



5(*S*)-lac-NAD$^+$

well as 5-methyl-NAD$^+$, is enzymatically active (Grau et al., 1978), indicating that there is indeed an accessible volume available in the active site around C-5 of the nicotinamide ring. The question to be considered by molecular modeling, is whether the 3-carboxamide or 3-formyl substituents are small enough to occupy this space without disrupting the relative conformation of the active site residues and substrates required for catalysis.

The crystal structure of the pig heart LDH complex with 5-(*S*)-lac-NAD$^+$ is not as highly refined as the structure of the dogfish muscle LDH, but the crystalline pig heart LDH is catalytically active and is the same enzyme we used experimentally. For computational purposes, lactate and NAD$^+$ were generated as separate structures by removing the methylene bridge from the 5-(*S*)-lac-NAD$^+$ in the crystal structure. Direct inspection of the positions of the active site residues indicates that there is a large unoccupied space around the bound nicotinamide ring which would allow the NAD$^+$ to bind in the syn conformation (Figure 2). The nearest residues to the carboxamide in the syn conformation are Gln-102 and Thr-246, which define a gap large enough for the carboxamide group to occupy. Gln-102 is part of the "mobile loop" of the enzyme which closes over the substrates in the ternary complex (White et al., 1976), so there may be significant conformational flexibility of this residue. Wilks et al. (1988) mutated the analogous glutamine in the *Bacillus stearothermophilus* LDH to an arginine and demonstrated that the mutant enzyme became an efficient malate dehydrogenase. The mutant enzyme presumably accommodates the extra bulk of the arginine side chain as well as the additional carboxyl of the substrate in the gap between Thr-246 and Gln-102 shown in Figure 2.

The crystal structure of the ternary complex of dogfish muscle LDH suggests that Thr-246 may, in fact, present a significant steric barrier to NADH being bound in a syn conformation. In this structure the glycosidic torsion angle of the NADH (defined as C-2, N-1, C-1′, O_4′) is −106° compared to the −81° torsion angle observed for the analogous glycosidic bond in the pig heart 5-(*S*)-lac-NAD$^+$ complex. This 25° difference in glycosidic torsion angles results in the

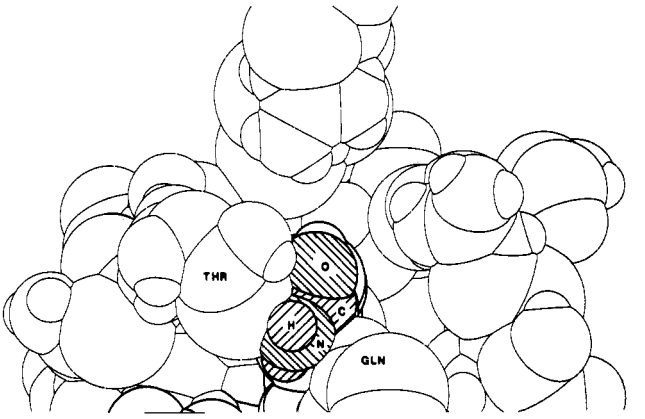


FIGURE 2: Space-filling view of NAD$^+$ in the syn conformation. The syn conformation was generated from the crystal structure (5LDH) by deleting the C-5 substituent from 5-(*S*)-lac-NAD$^+$ and rotating 180° about the glycosidic bond. Nonpolar hydrogens are included as extended carbon atoms with larger van der Waals radii. The carboxamide atoms are shaded and labeled with the appropriate element. Threonine-246 is labeled on the β-carbon and glutamine-102 on the γ-carbon. The largest steric overlap is between the extended methyl of threonine-246 and carboxamide nitrogen.

C-3 substituent of the nicotinamide ring being superimposed on the side chain of Thr-246 when it is rotated 180°. The possibility exists that in the 5-(*S*)-lac-NAD structure the nicotinamide ring has been forced to adopt a different glycosidic bond rotation so that the bridging methylene can occupy the gap between Thr-246 and Gln-102. Alternatively, the difference in torsion angle may reflect a difference between the oxidized and reduced coenzyme, with the oxidized coenzyme having a preference for a torsion angle closer to 0 (Wu & Houk, 1991). While using 5-(*S*)-lac-NAD may appear to have artificially induced a conformation capable of binding the syn nucleotide, the enzymatic activity of the bisubstitute analogue indicates that the glycosidic bond angle observed in the dogfish ternary complex is not uniquely active. Further, saturation-transfer ^{1}H-NMR experiments of NADH bound to liver LDH have been interpreted as suggesting that NADH is bound in both syn and anti conformations in the binary complex (J. Burgner, personal communication).

It is possible that the observed gap may close in the transition state, thus destabilizing the transition state with a syn conformation of the nicotinamide. However, this possibility is not supported by the observation that 5-methyl-NAD$^+$, 5-(*S*)-lac-NAD$^+$ (Grau et al., 1978), and long-chain keto acids (Holbrook et al., 1975) are all catalytically active substrates for this enzyme, which would not be possible if this gap had to close significantly during catalysis.

CHARMm (Brooks et al., 1983) energy minimizations, with the nicotinamide constrained to remain in the syn and anti conformations and with only those atoms within 10 Å of C-4 of the nicotinamide ring allowed to move, resulted in the syn conformation being calculated to be 4 kcal/mol more stable than the anti conformation. The quantitative accuracy of this calculation is less than might be accomplished using a more sophisticated level of modeling (e.g., quenched dynamics; Novotny et al., 1985). We proposed to answer only one question: Is there a major steric barrier that prevents the *binding* of NAD$^+$ in a syn conformation? The answer from the modeling is an unambiguous no.

Polarization of the 3-Carboxamide. If the extreme stereospecificity observed is not attributable to steric exclusion, what molecular interactions are responsible? The potential interactions of the carboxamide with the active site are shown in Figure 3. Our results with pyrAl-AD$^+$ require that any

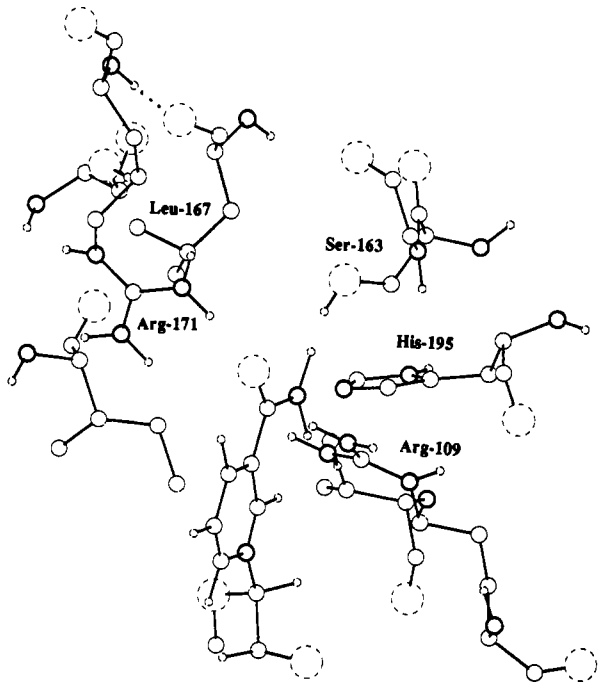


FIGURE 3: Active site environment of the carboxamide. The coordinates are from structure 5LDH in the Brookhaven Protein Data Bank. Carbon, nitrogen, oxygen, and polar hydrogens are represented as closed circles, bold circles, larger dashed circles, and small dashed circles, respectively. The lactate, which forms a salt bridge with Arg-171 and H-bonds to His-195, has been omitted. The hydrogen bond between the amide proton of Arg-171 and carbonyl of Leu-167 is shown as a dotted line at the upper left to emphasize the direction of the α2F helix.

energetically significant reactions be with the carbonyl of the C-3 substituent; hydrogen bonding to the amide nitrogen or protons cannot play a dominant role. An electron-withdrawing carbonyl at C-3 of the pyridine ring is necessary for activity with lactate dehydrogenase (Anderson, 1982; Samama et al., 1981). In Raman studies of NAD^+ (Deng et al., 1989a) and acetylpyridine adenine dinucleotide (Deng et al., 1989b) bound to lactate dehydrogenase, the carbonyl has been observed to be polarized by H-bonding to a solvent-exchangeable proton with an estimated interaction energy of 7.7 kcal/mol. In the dogfish muscle LDH ternary complex crystal structure, the carbonyl of the carboxamide is hydrogen bonded to a water molecule that potentially forms three additional hydrogen bonds (one as a donor and two as an acceptor). One of the hydrogen bonds is to Gly-146 at the amino terminus of the α2F helix. The carbonyl is just off the helix axis and may be polarized by interaction with the helical dipole (Hol, 1985). The macrodipole of the α helix effectively generates a $+0.5e$ to $+0.75e$ charge (Hol et al., 1978), depending on the effective local dielectric, at the helix amino terminus. The relative orientation of the carboxamide carbonyl and that of the α2F helix are shown in the stereodiagrams of Figure 4. In Figure 4A the carboxamide defines the xy plane and identifies the location of the carbonyl relative to the α2F helix. In Figure 4B, the structure has been rotated 90°. Gilson and Honig (1988) have shown that helical dipoles can exert an effect off the helix axis, particularly when the amino terminus is in the protein interior, as is the case with the α2F helix. In the structurally similar cytoplasmic malate dehydrogenase, the carbonyl oxygen of the 3-carboxamide is similarly hydrogen bonded to a water molecule at the amino terminus of the analogous α2F helix (Birktoft et al., 1989). Arg-109, Arg-171, and His-195 are other potential sources of positive charge that could polarize the carbonyl. Higher level calculations would be required to ascertain the contributions of each to the potential field that polarizes the carbonyl.

The isotope effects on the binding of NAD^+ to lactate dehydrogenase (LaReau et al., 1989) further support the polarization of the carbonyl and concomitant reduction of electron density of C-4 of the nicotinamide. For the most part, the role of the carboxamide substituent has been either ignored or given a static role in the various proposed enzymatic mechanisms of the dehydrogenases [e.g., Grau (1982) and Wilks et al. (1988)]. However, theoretical calculations (Donkersloot & Buck, 1981) and the reactions of model compounds led deKok et al. (1986) to propose that the out-of-plane rotation of the carboxamide arm stabilizes the transition state for hydride transfer by an electrostatic interaction of the carbonyl oxygen with the cosubstrate's carbonyl carbon. This interaction may be enhanced by the additional polarization imparted by the electrostatic interaction with the α2F helix. The simulated out-of-plane rotation of the C-3 substituent of NAD^+ analogues has been correlated with enzymatic activity (Beijer et al., 1990). An alternative source of the extreme stereospecificity may be that the active site of LDH, potentially His-195 and Arg-109, is capable of inducing a favorable out-of-plane rotation of the carboxamide in the anti conformation, but not in the syn conformation.

If the polarization of the carboxamide carbonyl is an intrinsic part of the catalytic mechanism utilized by LDH, then the stereospecificity may be understood in terms of stabilization of the *transition state* of the reaction only when the nicotinamide ring is placed in an anti conformation. This model is in contrast to the "lock and key" proposal that the enzyme's active site "arbitrarily" binds the cofactor in an anti (rather than a syn) conformation and that interactions with the car-

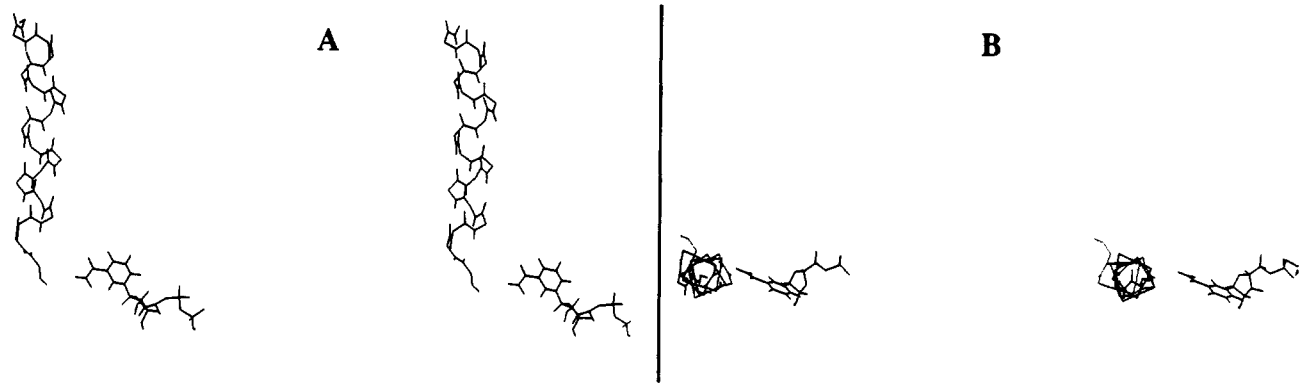


FIGURE 4: Stereoview of the orientation of NAD^+ and the α2F helix. The coordinates are from structure 4LDH in the Brookhaven Protein Data Bank. View A has the xy plane defined by the carboxamide of the nictonamide. View B is rotated 90° around the x-axis, giving a view down the carbon–oxygen double bond, which is barely visible since it deviates only slightly from the z-axis.

boxamide play no additional role in stabilizing the transition state.

Registry No. LDH, 9001-60-9; 3-pyrAlAD$^+$(H), 5263-47-8; NAD, 53-84-9; 3-pyrAlNAD$^+$, 86-07-7; lactic acid, 50-21-5; glycolic acid, 298-12-4.

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