acetate–acetonitrile (95:5)] retention time = 24 min at 1.5 mL/min. The peak coincided with that of unlabeled (3'→5')UpA: UV (H_2O) λ_{max} 259 nm.

(R_p)-[^{17}O,^{18}O]Uridylyl(3'→5')adenosine Ammonium Salt (7b). Using the same reaction conditions as described for compound **7a** and starting with **5b** (115 mg, 0.09 mmol), we obtained colorless fluffy **7b** (40 mg, 75%). UV and HPLC data were identical with those of the isomer **7a** and (3'→5')UpA. Enzymatic cleavage at 37 °C with spleen phosphodiesterase (0.05 M ammonium acetate, pH 6.5) furnished uridine 3'-monophosphate and adenosine (TLC, PEI–cellulose, solvent D); the same reaction products were obtained by hydrolysis with ribonuclease A (0.01 M Tris-HCl, pH 7.8 37 °C). Snake venon phosphodiesterase digested **7b** to adenosine 5'-monophosphate and uridine (0.01 M Tris-HCl, pH 9.0, 37 °C). Identical cleavage products as described for **7b** were also found for **7a** in all cases.

Configurational Analysis of (R_p)-[^{17}O,^{18}O]Uridylyl(3'→5')adenosine (7b). (a) Hydrolysis of (R_p)-[^{17}O,^{18}O]UpA by Nuclease P1 in $H_2{}^{17}O$. [^{17}O,^{18}O]UpA **7b** (42.8 μmol), derived by deprotection of **4b** (slower migrating zone), was dissolved in $H_2{}^{17}O$ (^{17}O, 52.8%; 100 μL) and left at room temperature for 30 min. The $H_2{}^{17}O$ was removed in vacuo under thoroughly anhydrous conditions, and the solid residue was left on a vacuum line for 15 h. The residue was then dissolved in $H_2{}^{17}O$ (350 μL) and nuclease P1 was added as a lyophilized powder (300 μg, ca. 120 units). The solution was incubated at 37 °C and the cleavage reaction monitored by HPLC. The reaction was seen to be essentially complete after 9 min, but the mixture was left for a total time of 30 min at 37 °C and then applied to a DEAE-Sephadex A-25 column (30 × 25 cm) and the reaction products were eluted with a gradient of 1 L each of 50 and 250 mM triethylammonium bicarbonate. Fractions of approximately 20 mL were collected. Uridine (37 μmol) was eluted in fractions 10–17 and [^{16}O,^{17}O,^{18}O]AMP **8** (35.6 μmol) in fractions 72–91. The latter fractions were pooled and evaporated to dryness in vacuo, excess triethylammonium bicarbonate being removed by the evaporation of several volumes of methanol.

(b) Configurational Analysis of (S_p)-[^{16}O,^{17}O,^{18}O]AMP (8). [^{16}O,^{17}O,^{18}O]AMP triethylammonium salt (35.6 μmol) was converted to the tri-*n*-octylammonium salt via the pyridinium salt and cyclized with diphenylphosphochloridate and potassium *tert*-butoxide to the isotopomers of [^{16}O,^{17}O,^{18}O]cAMP by the method of Jarvest et al.[10a] Purification by DEAE-Sephadex chromatography as described above gave 7.7 μmol (22%) of pure [^{16}O,^{17}O,^{18}O]cAMP, which was eluted in fractions 15–22. After evaporation of solvent in vacuo and conversion to the potassium-18-crown-6 salt, this product was methylated by using methyl iodide in Me_2SO according to the method of Jarvest et al.[10a] The ^{31}P NMR spectra obtained are shown in Figure 3: ^{31}P NMR (Me_2SO-d_6–Me_2SO, 50:50 v/v) δ −2.809, −2.827, −2.851, −2.869 (4 s) equatorial series; −4.032, −4.046, −4.074, −4.087, (4 s) axial series (referenced to H_3PO_4).

(R_p,S_p)-Uridylyl(3'→5')-N^1-methyladenosine Methyl Ester (9a/b). UpA ammonium salt (7 mg, 11.9 μmol) was dissolved in water (5 mL) and the solution stirred with Dowex 50 XW (1 mL, potassium form) for 30 min. The solution was filtered from the resin, and filtrate and washings were evaporated to a small volume. 18-Crown-6 was added (15 mg, 57 μmol) and the resulting solution evaporated thoroughly to dryness in vacuo. The residue was dissolved in dry DMF (5 mL), which was evaporated under anhydrous conditions. This was repeated 3 times. The residue was finally dissolved in Me_2SO-d_6 (200 μL) and methyl iodide (100 μL) was added. After the mixture had been stirred for 12 h the methyl iodide was removed in vacuo to give (R_p,S_p)-uridylyl(3'→5')-N^1-methyladenosine methyl ester as a solution in Me_2SO-d_6. The methylation was effectively quantitative. To this solution were added a few crystals of 8-hydroxyquinoline, followed by methanol (200 μL). The resulting solution was filtered and the ^{31}P NMR spectrum recorded (Me_2SO-d_6–MeOH, 50:50 v/v) δ −3.11 (s) for the S_p diastereoisomer and −3.21 (s) for the R_p diasteroisomer (with trimethyl phosphate as the external standard) (Figure 4a).

(R_p,S_p)-[^{17}O,^{18}O]Uridylyl(3'→5')-N^1-methyladenosine Methyl Ester (10a/b). [^{17}O,^{18}O]Uridylyl(3'→5')-N^1-methyladenosine methyl ester was synthesized from (R_p)-[^{17}O,^{18}O]UpA (9.2 μmol) exactly as described for the unlabeled compound. ^{31}P NMR (Me_2SO-d_6–MeOH, 50:50 v/v) δ −3.139, −3.180 (2 s) and −3.237, −3.253 (2 s) (with trimethyl phosphate as the standard) (Figure 4b).

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Note Added in Proof. (a) In the dinucleoside monophosphates and their protected derivatives, the nomenclature [^{17}O,^{18}O] is intended to signify the presence of both isotopes at one position at phosphorus. (b) The phosphorus decoupled ^{17}O NMR spectra of **7a** and **7b** gave both singlets at 93.04 ppm (water, pH 6.1) [Gerlt, J. A., private communication].

A Mechanistic Basis for the Stereoselectivity of Enzymatic Transfer of Hydrogen from Nicotinamide Cofactors

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Abstract: A mechanistic theory explains the stereochemical preferences of dehydrogenases dependent on nicotinamide cofactors that interconvert alcohols and ketones. This theory is based on the principles of stereoelectronic control and assumes that the Gibbs free energies of reactants and products are more nearly matched when bound in the active site of an optimal enzyme than in solution. This theory makes experimentally testable predictions both of the stereochemical preferences of dehydrogenases and of the nature of the free energy profile of enzyme-catalyzed reactions. Consistent with these predictions, we report that the enzyme lactaldehyde reductase (EC 1.1.1.55) from pig kidney catalyzes the transfer of the *pro-R* (A) hydrogen from NADH, and we provide estimates for the "internal" equilibrium constant between the ternary complexes enzyme–alcohol–NAD^+ and enzyme–ketone–NADH.

Thirty years ago, in a classical demonstration that enzymes could distinguish between enantiotopic groups on substrate molecules, Westheimer and Vennesland showed that dehydrogenases catalyze the stereoselective transfer of hydrogens from the 4-position of reduced nicotinamide cofactors.[1] Subsequent studies showed that dehydrogenases appeared to be distributed "randomly" between two stereochemical classes, those transferring the *pro-R* hydrogen and those transferring the *pro-S* hydrogen.[2] These observations present the most puzzling problem

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Table I

enzyme[a]	EC no.	$-\log E_{eq}$[b]	stereo-chem-istry[c]
glyoxylate reductase	1.1.1.26	17.5	*pro-R*
glyoxylate reductase ($NADP^+$)	1.1.1.79	17.5	*pro-R*
tartronate-semialdehyde reductase	1.1.1.60	13.3	*pro-R*
glycerate dehydrogenase[d]	1.1.1.29	13.3	*pro-R*
glycerol 1-dehydrogenase	1.1.1.72	12.8	*pro-R*
hydroxypyruvate reductase ($NADP^+$)[d]	1.1.1.81	12.4	*pro-R*
malate dehydrogenase	1.1.1.37	12.1	*pro-R*
malate dehydrogenase ($NADP^+$)	1.1.1.82	12.1	*pro-R*
malic enzyme	1.1.1.38	12.1	*pro-R*
malic enzyme ($NADP^+$)	1.1.1.40	12.1	*pro-R*
L-lactate dehydrogenase	1.1.1.27	11.6	*pro-R*
D-lactate dehydrogenase	1.1.1.28	11.6	*pro-R*
ethanol dehydrogenase (yeast)	1.1.1.1	11.4	*pro-R*
glycerol 2-dehydrogenase	1.1.1.6	11.3	*pro-R*
glycerol-3-phosphate dehydrogenase	1.1.1.8	11.1	*pro-S*
homoserine dehydrogenase	1.1.1.3	10.9	*pro-S*
carnitine dehydrogenase	1.1.1.108	10.9	*pro-S*
3-hydroxyacyl-CoA dehydrogenase	1.1.1.35	10.5	*pro-S*
3-hydroxybutyrate dehydrogenase	1.1.1.30	8.9	*pro-S*
3β-hydroxysteroid dehydrogenase	1.1.1.50	8.0	*pro-S*
testosterone dehydrogenase	1.1.1.64	7.6	*pro-S*
3-oxoacyl-ACP dehydrogenase	1.1.1.100	7.6	*pro-S*
β-Hydroxysteroid dehydrogenase	1.1.1.51	7.6	*pro-S*
oestradiol 17β-dehydrogenase	1.1.1.62	7.7	*pro-S*

[a] The nomenclature of the Enzyme Commission is used; equilibrium constants are used directly from the literature and often vary depending on the precise conditions used. [b] K_{eq} is defined as [ketone] [NADH] [proton] /([alcohol] [NAD^+]). [c] The *pro-R* hydrogen is classically refered to as the "A" hydrogen; the *pro-S* hydrogen is the "B" hydrogen. [d] Two equilibrium values for the same basic reaction are reported.

in enzyme stereochemistry: is there any explanation for the stereochemical choices made by dehydrogenases? Thirty years of study and conjecture has failed to yield a plausible answer to this question.[2]

We now offer a solution to this puzzle. Our solution is based on the observation that, for enzymes catalyzing the redox reaction between carbonyls and their corresponding alcohols, *thermodynamically unstable carbonyls are reduced with the pro-R hydrogen of NADH, while thermodynamically stable carbonyls are reduced with the pro-S hydrogen.*[3] When the equilibrium constant between ketone and alcohol is used as a measure of the stability of a carbonyl substrate, the correlation between the stability of the carbonyl group and the stereochemical preference of the enzyme that has evolved to reduce it is quite striking (Table I).[4]

Our solution has a mechanistic basis, resting on four hypotheses:[3] (1) The *pro-R* hydrogen is transferred from a nicotinamide cofactor bound in the active site in an anti conformation; the *pro-S* hydrogen is transferred from a nicotinamide cofactor bound in a syn conformation.[5] (2) *anti*-NADH is a weaker reducing agent than *syn*-NADH. (3) Optimal enzymes bind substrates so as to match or nearly match the free energies of bound intermediates.[6] (4) Dehydrogenases have evolved to be optimal enzymes.

These hypotheses together provide a mechanistic basis for the correlation shown in Table I. Remarkably, the stereochemical preferences of dehydrogenases appears to depend on principles of "stereoelectronic control",[7-9] a controversial principle of "matched internal thermodynamics",[6,10,11] and the notion that these dehydrogenases have evolved to be evolutionarily optimal, where evolutionary optimality is defined in a purely chemical sense. *This combination of fundamental chemical principles, thermodynamics, and evolutionary rationale to explain a series of stereochemical choices of proteins is unprecedented in enzymology.*

Most importantly, the hypotheses that comprise our proposed solution are experimentally testable. They predict the stereochemical preference for any dehydrogenases acting to reduce a carbonyl whose redox potential is known. Furthermore, they predict that the equilibrium constant between enzyme-bound cofactor and substrates should be closer to unity than the equilibrium constant between corresponding states in solution.

We report here the first experimental verifications of predictions made by our proposal: the stereochemical preference of the enzyme lactaldehyde dehydrogenase and magnitude of the internal equilibrium constant for three dehydrogenases. As the equilibrium constant for the reaction catalyzed by lactaldehyde reductase (2.4×10^{-13}) is well into the *pro-R* "region" in Table I, we predicted that the enzyme should catalyze the selective transfer of the *pro-R* hydrogen. Furthermore, we have gathered evidence supporting our prediction that the ratio at equilibrium of the ternary complexes enzyme–NAD^+–alcohol and enzyme–NADH–carbonyl is in fact much closer to unity than that between alcohol and NAD^+ vs. carbonyl and NADH in solution.

Experimental Section

Preparation of Materials. Lactaldehyde reductase (EC 1.1.1.55) was purified from frozen pig kidney by the method of Robinson.[12] The catalytic activity of the enzyme was measured in 20 mM potassium phosphate buffer at pH 7.4 by spectroscopic measurement of the loss of absorbance at 340 nm due to consumption of NADPH in the presence of lactaldehyde. Purified enzyme has specific activities of 1–2 units/mg. As the enzyme loses activity over a period of weeks even when stored frozen, preparations were either used immediately or after only a short period of storage at −10 °C.

Racemic lactaldehyde was prepared by reduction of pyruvaldehyde dimethyl acetal (Aldrich) with sodium borohydride in methanol to form the dimethyl acetal of lactaldehyde, which was purified by vacuum distillation and hydrolyzed in 50 mM phosphate buffer at pH 7.0. 1,2-Dihydroxypropane was a generous gift of Stephen Buchwald and J. R. Knowles. [4′(*S*)-^{3}H]NADPH was prepared by the stereospecific reduction of $NADP^+$ with [1-^{3}H]glucose 6-phosphate catalyzed by glucose 6-phosphate dehydrogenase (Sigma). Tritiated glucose 6-phosphate was prepared from [1-^{3}H]glucose (New England Nuclear, 35–40 Ci/mmol), with hexokinase (Sigma) and ATP. The glucose 6-phosphate was purified by ion-exchange chromatography on Dowex 1 with dilute formic

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(4) We have included in the table every enzyme reducing a simple, unconjugated ketone or aldehyde where both the stereochemical preference and the external equilibrium constant for the natural substrate is known. For further discussion about the selection of enzymes, see ref 3.

(5) Stereoelectronic principles provide for the theoretical basis for this hypothesis. The dihydronicotinamide ring should be distorted into a boat conformation, so as to maximize the overlap between the lone pair of electrons on nitrogen and the antibonding orbital associated with the ribose carbon–oxygen bond. This distortion places the *pro-R* hydrogen axial when the cofactor is in the anti conformation and the *pro-S* hydrogen axial when in the syn conformation. Evidence for this boat conformation is available from crystallographic studies, and further support for this hypothesis can be obtained from crystallography. (See ref 3.)

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(11) For a contrasting case where the energies of enzyme-bound substrates do *not* appear to be matched, see: Rahil, J. F.; de Maine, M. M.; Benkovic, S. J. *Biochemistry* **1982**, *21*, 3358–3363.

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Table II

enzyme	substrates	NADH/NAD$^+$ (bound)[a]	conditions	K_{eq}-(external)[b] × 10^4
lactate dehydrogenase	pyruvate–lactate	1.5 ± 0.5	5 °C, pH 7.5	1
yeast alcohol dehydrogenase	CH_3CHO–CH_3CH_2OH	0.2 ± 0.05	25 °C, pH 7.25	1.4
horse liver alcohol dehydrogenase	CH_3CHO–CH_3CH_2OH	0.15 ± 0.05	25 °C, pH 7.3	2
	cyclohexanone–cyclohexanol	1.0 ± 0.5	25 °C, pH 7.65	
	acetone–2-propanol	3.5 ± 1	25 °C, pH 7.6	

[a] Best results from four or more runs. [b] Calculated K_{eq}(external) = NADH/NAD$^+$ with a 1 molar standard state in both substrates at the pH of enzymic measurement, from literature values.

acid as an eluant. [4′(*R*)-^{3}H]NADPH was prepared by stereospecific reduction of NADP$^+$ with [α-^{3}H]benzyl alcohol catalyzed by horse liver alcohol dehydrogenase (Sigma). Tritiated benzyl alcohol was prepared from [1-^{3}H]benzaldehyde by reduction with sodium borohydride. Tritiated benzaldehyde was prepared via the cyanide-catalyzed exchange of the aldehyde proton of benzaldehyde with tritiated water (New England Nuclear). The radioactive benzyl alcohol was purified by thin-layer chromatography.

NAD$^+$, NADP$^+$, NADH, and NADPH were all purified by chromatography on DEAE-cellulose.[13] The reduced cofactors are known to decompose rapidly in aqueous solution, and all preparations were used within 12 h of purification. Enzymatic and spectrophotometric assays were routinely used to confirm the purity of the products.

Stereospecificity of Lactaldehyde Reductase. To determine the stereochemical course of the reaction catalyzed by lactaldehyde reductase, mixtures containing enzyme, lactaldehyde, and either *pro-R* or *pro-S* tritiated NADPH were incubated in parallel runs in phosphate buffer (50 mM, pH 7.0) at 25 °C. The progress of the reaction was followed in a parallel reaction mixture identically prepared but using unlabeled cofactor. After approximately 20% conversion, the mixture was quenched and diluted with carrier 1,2-dihydroxypropane, and the 1,2-dihydroxypropane was isolated by ether extraction after the incubation mixture was passed through a column of Dowex 1 ion-exchange resin. The crystalline diphenylurethane of 1,2-dihydroxypropane was prepared by reacting the compound with phenyl isocyanate, and radioactive samples of this derivative were recrystallized from ethanol to constant specific activity.[14] The samples were then counted in Liquifluor in a liquid scintillation counter.

Measurement of Internal Equilibria. Enzymes were purchased from Sigma and assayed to determine their specific activities and to demonstrate the absence of any contaminating cofactors. Equilibrium measurements were made in phosphate buffer (50 mM) at the indicated pH and temperature (Table II), and the pH was measured before and after each set of experiments.

These experiments are based on the fact that virtually *all* cofactor will be *enzyme bound* in a solution containing a stoichiometric excess of enzyme over cofactor if the concentration of enzyme is large compared to the dissociation constants of the cofactors. To ensure that most (>90%) of the cofactor was bound to the enzyme, the concentration of enzyme in these experiments was typically more than twice the concentration of substrate and at least 10 times the dissociation constants for the least tightly bound cofactor (in these cases, NAD$^+$). Control experiments were run at varying concentrations of enzyme, cofactor, and substrate to demonstrate that, in fact, most of the cofactor was enzyme bound under the experimental conditions.[15]

When enzyme–cofactor complexes are immersed in a redox buffer of alcohol and ketone substrates at concentrations that are large compared to their respective dissociation constants from enzyme–cofactor complexes, a mixture of the ternary complexes (enzyme–NADH–ketone, enzyme–NAD$^+$–alcohol, enzyme–NADH–alcohol, and enzyme–NAD$^+$–ketone) is formed. As the latter two complexes are "abortive", we assumed that they are formed to a smaller extent than the "productive" complexes.

Because all cofactor under these conditions is bound to enzyme in a ternary complex, any ultraviolet absorbance arising from cofactor arises from bound cofactor. As enzyme-bound NADH absorbs much more intensely than enzyme-bound NAD$^+$ at 340 nm, the relative amounts at equilibrium, and hence the internal equilibrium constant between the two ternary complexes, enzyme–NAD$^+$–alcohol and enzyme–NADH–ketone, can be estimated from the ultraviolet absorbances of mixtures of enzyme, oxidized and reduced cofactor, and oxidized and reduced substrate at equilibrium.[18]

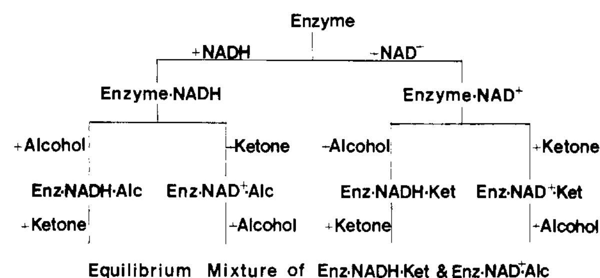

Figure 1. To a blanked cuvette is added enzyme. The absorbance of pure enzyme is noted and then either oxidized or reduced cofactor. The absorbance of enzyme–NAD$^+$ and enzyme–NADH is again noted, providing the range of optical densities where the fraction of NADH is 0% and 100%, respectively. Then, either ketone or alcohol is added to each cuvette, providing absorbances of the "abortive" complexes (enzyme–NAD$^+$–ketone or enzyme–NADH–alcohol, again with the fraction of NADH 0% or 100%, respectively) or the "single turnover complexes", yielding predominantly the "productive complexes" (enzyme–NAD$^+$–alcohol and enzyme–NADH–ketone). Finally, the remaining component of the redox buffer (ketone or alcohol) is added to achieve the same equilibrium mixture consisting predominantly of the two productive complexes.

Experimentally, the equilibrium between these two ternary complexes was approached in four directions (Figure 1) by changing the sequence in which the four components of the equilibrium, NAD$^+$, NADH, alcohol, and carbonyl, were added to a cuvette containing a concentrated solution of enzyme. Absorbances at 340 nm of the enzyme–NADH–alcohol complex (all of the cofactor reduced, the maximum absorbance) were obtained from mixtures of enzyme, NADH, and alcohol. Absorbances at 340 nm for the enzyme–NAD$^+$–ketone complex (all of the cofactor oxidized, the minimum absorbance) were obtained from mixtures of enzyme, NAD$^+$, and ketone. In each case, the completion of the redox buffer (ketone and alcohol) produced the same final equilibrium position.

Results

The results from the stereochemical experiments are unambiguous. The diphenylurethane of propanediol prepared by reduction of lactaldehyde with [4′(*R*)-^{3}H]NADPH, after dilution with carrier, contained a constant 600 cpm/g of radiolabel and melted sharply at 145–146 °C.[14] Propanediol prepared by reduction with [4′(*S*)-^{3}H]NADPH contained no radioactivity. Thus, as predicted, lactaldehyde reductase from pig kidney specifically transfers the *pro-R* hydrogen of NADPH.

Internal equilibria for three hydrogenases are shown in Table II. As has been previously observed,[16] the absorption maximum of enzyme-bound NADH is shifted slightly in comparison with NADH in aqueous solution. With "alcohol" dehydrogenase from horse liver, where the "natural" substrate is not known, the "internal" equilibrium constant varies with the nature of the ketone/alcohol parallel with the "external" equilibrium constants in these systems.[17] More important to the hypotheses presented above is the equilibrium constant calculated for ethanol de-

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hydrogenase from yeast and lactate dehydrogenase from rabbit muscle. Under the experimental conditions, the external equilibrium constants ($NADH/NAD^+$) for the reactions catalyzed by the two enzymes were approximately 0.0001; however, the equilibrium constants between the same species bound in the active sites were between 0.2 and 2.0.

Discussion

The correlation in Table I applies to enzymes that meet the following three criteria: the enzyme must catalyze the interconversion of "simple" unconjugated carbonyls with their corresponding alcohols, its natural substrate must be well-defined, and the equilibrium constant for the overall reaction of that substrate must lie at least 1 log unit away from the position of the "break" between *pro-R-* and *pro-S*-specific enzymes. Any enzyme conforming to these criteria and not fitting the correlation we consider a violation of the correlation, necessarily prompting the reevaluation of the correlation and the theories supporting it.

Lactaldehyde reductase is, of course, only a single enzyme. However, the experimental determination of its stereospecificity is a singularly important event in enzyme stereochemistry, as it represents *the first time a mechanistically based theory has ever successfully predicted the stereochemical preference of a dehydrogenase.* We encourage others to test our theory by examining the stereochemical preferences of enzymes that meet the three criteria listed above, and we will continue to do so ourselves.

The method that we have developed for measuring the internal equilibria for dehydrogenases should have broad applicability. It is an extension of an observation made by Theorell over 30 years ago[18] and follows qualitatively from the fact that most dehydrogenases bind reduced cofactor more tightly than oxidized cofactor and ketones and aldehydes more tightly than their corresponding alcohols. Alternative methods for measuring the magnitude of internal equilibrium constants include NMR,[10a] stopped flow,[10c] or rapid quench[10b] techniques. Alternatively, detailed kinetic studies can occasionally provide estimates for the magnitude of microscopic rate constants connecting two enzyme states and hence the equilibrium between these states.[10d] The principle weakness of our method, which is shared by other equilibrium methods and, in most cases, by kinetic methods, is that multiple forms of enzyme-bound cofactor (including abortive complexes) are all counted together in a single spectroscopic measurement. Furthermore, the equilibrium constant can only be measured accurately if it is close to unity. However, our method does not require successful rapid quenching of enzyme–substrate complexes.

The predictive success of the four mechanistic hypotheses listed in the introduction to this paper has implications well beyond their value as a solution to a particular stereochemical problem in enzymology. The structural arguments (hypotheses 1 and 2) require application of molecular orbital arguments often collected under the rubric of the "anomeric effect" (Figure 2). The anomeric effect was first identified by Altona[7] and Lemieux[8] and has met with singular success, predicting the stereochemical features of organic reactions in solution.[8] In its latter capacity, the molecular orbital arguments are often referred to collectively as principles of "stereoelectronic control". Although widely understood among synthetic and physical organic chemists, these principles have only been rarely applied to enzymes.[19] The predictive success of a theory based on stereoelectronic considerations suggests a general rule: *stereoelectronic considerations should be considered as potentially governing features in the design of the active sites of enzymes.*

Furthermore, if our rationale is correct, it represents a remarkable example of convergent evolution, in which dehydrogenases isolated from diverse segments of life all follow a mechanistic imperative to produce a single pattern of stereochemical results. The possibility that such a mechanistic imperative exists in other enzymes was recognized by Hanson and Rose some time ago.[20] However, unlike in the cases these authors considered, it is improbable that the stereoselectivity of dehydrogenases can be explained by evolutionary conservation of the stereopreferences of ancestral primordial enzymes.[21] Thus, predictive success of our four hypotheses, especially if it is extended to enzymes drawn from organisms having widely disparate evolutionary backgrounds, suggests another general rule: *stereochemical heterogeneity in this class of enzymes may reflect the evolution of enzymes to become highly refined catalysts.*

(19) Dunathan, H. C.; Voet, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3888–3891. Benner, S. A.; Morton, T. H. *J. Am. Chem. Soc.* **1981**, *103*, 991–993.

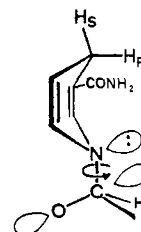

Figure 2. In a "reverse anomeric effect", the donation of electron density from the lone pair on nitrogen into the adjacent antibonding orbital of the sugar oxygen–carbon bond is optimized by distortion of the reduced nicotinamide ring into a boat conformation. This makes the *pro-R* hydrogen at the 4-position axial when the cofactor is in the "anti" conformation and the *pro-S* hydrogen axial when in the "syn" conformation. Principles of orbital overlap suggest that the axial hydrogen is the hydrogen most easily transferred. One testable implication of the donation of electron density into the adjacent antibonding orbital is that it gives the anomeric carbon–nitrogen bond some double-bond character. Thus, rotation around the glycosidic bond in the reduced cofactor should be slower than in the oxidized cofactor.

Why Are Internal Equilibria Close to Unity? It appears to be a general rule that enzyme-bound ternary complexes, such as enzyme–NAD^+–alcohol and enzyme–NADH–ketone, have energies more nearly equal than those of similar species in solution.[10] In addition to dehydrogenases, internal equilibria in kinases and isomerases also appear to be close to unity,[10] although there appear to be cases where this thermodynamic phenomenon is not observed.[11] This phenomenon was clearly anticipated in a seminal paper by Albery and Knowles in 1976,[6] a contribution that has been widely acknowledged in the literature.[10] In some circles, the Albery–Knowles argument has even been interpreted as showing conclusively that internal equilibria close to unity are *required* of all enzymes that have evolved to evolutionary "perfection".[10d] "Matched internal thermodynamics" may therefore be the "mechanistic imperative"[20] that dehydrogenases are responding to as they evolve to transfer hydrogens stereospecifically. In view of the data collected by us and others, it is clearly opportune to consider here in a rigorous fashion what the physical basis for that imperative might be and to what extent existing treatment of the phenomena is valid or even reasonable.

Albery and Knowles' argument[6] that the free energies of enzyme-bound species in an evolutionarily optimal enzymes are matched is based on four assumptions: (1) The evolutionary optimal enzyme is the enzyme that catalyzes the conversion of substrate to product at the diffusion-limited rate. (2) Enzymes conform to a "uniform binding criterion", evolutionarily adjusting free energy profiles most rapidly by moving the energies of bound states *up* and *down together.* (3) There exists a linear relationship between rate and equilibrium of a reaction, analogous to a Bronsted linear free energy relationship, described by the equation

$$\ln k = \ln k_{K_{eq}=1} + \beta \ln K_{eq} \quad (1)$$

(4) The magnitudes of various rate constants are in certain ranges. The reader is referred to their original paper[6] for a complete discussion of these assumptions and their justification.

From these assumptions, Albery and Knowles[6] derived a relationship between the equilibrium constant for an internal re-

(20) Hanson, K. R.; Rose, I. A. *Acc. Chem. Res.* **1975**, *8*, 1–14.

(21) Both by sequence and from the existence of a "nucleotide binding fold", *pro-R* and *pro-S*-specific dehydrogenases all appear to be interrelated, yet a single stereochemical preference has been conserved throughout evolution for enzymes reducing a particular substrate.

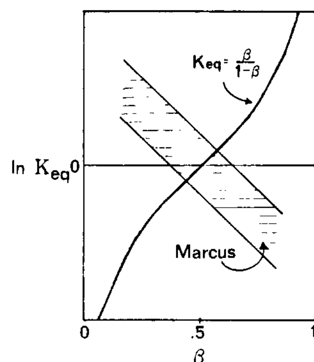

Figure 3. The ascending line describes the relationship between the equilibrium constant (K_{eq}) and the Bronsted β for an "optimal" enzyme.[5] The shaded area between the two descending lines represent pairs of β and K_{eq} found in nature, as predicted by the Marcus theory for group transfer. If an enzyme selects an equilibrium constant that is both optimal (falling on the $K_{eq} = \beta/(1 - \beta)$ line) and real (falling in the shaded area), it must necessarily select an equilibrium constant (K_{eq}) approximately equal to unity.

action, K_{eq}, and the β of the linear free energy relationship written above:[22]

$$K_{eq} = \beta/(1 - \beta) \quad (2)$$

The Albery–Knowles interpretation of this equation is criticizable,[22,23] but the equation itself is remarkable. It describes an *increasing* function of K_{eq} with β; large β's give rise to large equilibrium constants and vice versa. This is precisely *opposite* of the *decreasing* relationship between K_{eq} and β that is qualitatively predicted by the Hammond postulate and quantitatively predicted by Marcus theory[24] for group transfer reactions. This suggests a simple explanation for the prevalence of matched energies of enzyme–substrate and enzyme–product complexes: matching the energies of bound states simultaneously satisfied the Albery–Knowles relationship (eq 2) and the Marcus relationship, providing a free-energy profile that is both evolutionarily optimal and consistent with the Hammond postulate (Figure 3).

This explanation is only valid to the extent that the assumptions used in the derivation of eq 2 are valid. Especially problematic is the first assumption, that "perfect" enzymes are diffusion limited. While the predictive success of our stereochemical explanation based on this assumption is tantamount to support of this assumption, there is no reason a priori to believe that microscopic rate constants are the parameters of an enzymatic reaction that are being optimized by evolutionary selective pressures. Indeed, Albery and Knowles themselves indicate that their analysis is appropriate only for enzymes mediating "constant concentrations" of their substrates and not to those enzymes creating a "constant flux" of substrate to products. They explicitly rule out regulated enzymes. It is not clear whether to expect enzymes outside the scope of the Albery–Knowles analysis to bind substrates and products to produce complexes having nearly equal free energies. Furthermore, it is clear that even for enzymes conforming rigorously to the conditions set forth in the Albery–Knowles analysis, diffusion-limited enzymes need not be evolutionarily optimal.[25] Finally, especially for enzymes operating primarily in one direction under physiological conditions, two alternative free energy profiles, one having the energies of enzyme–substrate complexes precisely matched and another having the energies of the complexes, reflecting somewhat the relative inherent stabilities of the substrate and product species (with the free energies of consecutive complexes along the reaction coordinate arranged as steps in a staircase), while theoretically quite different, may be within the experimental error in systems that have commonly been studied.

We make these points both to alert the chemical community to the existence of an interesting and potentially important problem arising from what are basically empirical generalizations about free energy profiles in enzymic catalysis and to note that the Albery–Knowles explanation is a fascinating introduction, not a definitive conclusion, to this problem. The application of "matched internal thermodynamics" to solve the stereochemical problem presented by dehydrogenases was unanticipated, to say the least, and the experimental verification of the solution by the data reported here should further stimulate serious thought about the nature of free energy profiles in enzymic catalysis, the importance of conformation and orbital overlap in the design of enzymic active sites, and the nature of evolutionary selection in catalytic proteins.

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Registry No. NADH, 58-68-4; NADPH, 53-57-6; dehydrogenase, 9035-82-9; EC 1.1.1.55, 9028-43-7; EC 1.1.1.26, 9028-32-4; EC 1.1.1.79, 37250-17-2; EC 1.1.1.60, 9028-68-6; EC 1.1.1.29, 9028-37-9; EC 1.1.1.72, 37250-11-6; EC 1.1.1.81, 9059-44-3; EC 1.1.1.37, 9001-64-3; EC 1.1.1.82, 37250-19-4; EC 1.1.1.38, 9080-52-8; EC 1.1.1.40, 9028-47-1; EC 1.1.1.27, 9001-60-9; EC 1.1.1.28, 9028-36-8; EC 1.1.1.1, 9031-72-5; EC 1.1.1.6, 9028-14-2; EC 1.1.1.8, 9075-65-4; EC 1.1.1.3, 9028-13-1; EC 1.1.1.108, 9045-45-8; EC 1.1.1.35, 9028-40-4; EC 1.1.1.30, 9028-38-0; EC 1.1.1.50, 9028-56-2; EC 1.1.1.64, 9028-63-1; EC 1.1.1.100, 37250-34-3; EC 1.1.1.51, 9015-81-0; EC 1.1.1.62, 9028-61-9.

(22) Algebraic problems encountered in the derivation of the Albery–Knowles relationship (eq 2) include the choice of a different function to optimize in the Appendix of their paper (ref 6a) than in the text and the relaxation of the constraint imposed to obtain the "uniform binding criterion" (eq 12 of the text of their paper) in subsequent optimization described in the Appendix. The latter is probably justified by the Albery–Knowles model of evolution, where "uniform binding" optimization occurs rapidly between events involving "differential binding" optimization, while the former does not appear to alter the principal thrust of their argument.

(23) For example, the argument that Albery and Knowles use to deduce "matched internal thermodynamics" from eq 2 appears to us to be decidedly circular. They deduce an internal K_{eq} of 0.4–2.5 (and therefore 1) by assuming that "most reactions" have values for β between 0.3 and 0.7. However, by the Hammond postulate, this assumption is tantamount to an assumption that the internal K_{eq}'s are approximately unity.

(24) Marcus, R. A. *J. Phys. Chem.* **1968**, *72*, 891–899.

(25) To illustrate this point, consider a biological system forced by selection pressure to select between two hypothetical enzymes catalyzing the turnover of a vital metabolic intermediate. A diffusion-limited enzyme is available and is of a size such that it costs an organism 1000 ATP units to synthesize it. An alternative, smaller enzyme also catalyzes the same reaction. It is slower, catalyzing the reaction at half of the diffusion limit, but it costs only 250 ATP units to synthesize. Were evolutionary pressure forcing the selection of enzymes in a fashion so as to minimize the consumption of ATP by the organism, the second, slower enzyme would be evolutionarily preferable to the faster, diffusion-limited enzyme.

(26) Experiments were run in 50 mM potassium phosphate buffer at the indicated pH and temperature. In several cases, the temperature was kept low to slow the rate of formation of covalent substrate–cofactor adducts. Enzyme concentrations were 10 times the dissociation constant for the least tightly bound cofactor, and substrate concentrations were 10 times the dissociation constant of the substrate. Both cofactors were rigorously purified by ion-exchange chromatography on DEAE-cellulose and used within 12 h of purification. Enzymes were commercially obtained from Sigma; controls were run to eliminate the possibility of significant amounts of endogenous cofactor bound in the commercial preparation. The measured equilibrium constants were largely (to within 5%) independent of the concentrations of oxidized and reduced substrate in the redox buffer; the slight dependence observed was consistent with expectations based on the fact that the substrate concentrations were only 10 times their dissociation constants and thus the variation could be corrected for. Blanks were independently measured for all substrates; with the exception of pyruvate, the substrate blanks were negligible. Calculation of these values assumes that the cofactor is 100% NADH when the only material added in NADH and reduced substrate; likewise, we have assumed that the appropriate "blank" is that containing only NAD and oxidized cofactor. We have also assumed in these calculations that there is no significant change in these values resulting from the addition of a second substrate. Of course, the principal weakness of this method (as with quench and NMR methods for determining the same internal equilibrium constant) is that all enzyme-bound species containing NADH contribute to the same measured absorbance, whether or not they are part of the reaction coordinate. We cannot exclude the possibility of any abortive ternary complexes contributing to the overall absorbances measured, a weakness shared by other methods for measuring internal equilibrium constants.