to be again the same as in the native protein (i.e., the upper orientation in Figure 1D). Saturation of the Ile-99 γ-CH_3 at variable temperature (not shown) again yields a peak with anti-Curie behavior that can be assigned to the 3-CH_3. The shifts for all heme methyls are listed in Table I.

Inter-Methyl NOE. The NOE between methyl groups attached to the same pyrrole or adjacent to a same meso position is consistent with a simple free-rotation model. In the 2,4-dimethyldeuterohemin-reconstituted metMbCN, the saturation of the 2-CH_3 causes an $\sim$-3.5% intensity change of the 1-CH_3 signal; this corresponds to an $\sim$-1.2% effect per irradiated proton. The intrinsic relaxation rate of the 1-CH_3 (peak c, Figure 3A) was measured to be 11.7 Hz under the same conditions, and thus eq 1 leads to $\sigma = -0.14$ Hz. This cross-relaxation rate implies (via eq 2 and a τ_c value of 7 ns) an experimental inter-methyl distance of $\sim$3.8 Å, while calculation of the average 1-CH_3 to 2-CH_3 distance $\langle r \rangle = \langle 1/r^3 \rangle^{1/3}$ using X-ray coordinates adjusted for the presence of the 2-CH_3 yields a value of $\sim$3.7 Å. On the other hand, the NOE from the 8-CH_3 to the 1-CH_3 is -1.0 ± 0.4%, which translates into an experimental separation of 4.7 ± 0.4 Å. This result is also comparable to the calculated average distance between the freely rotating 1-CH_3 and 8-CH_3, estimated at $\sim$5.1 Å from X-ray data.[10]

Conclusions

The homonuclear Overhauser effect has allowed us to assign all heme methyl resonances in sperm whale metMbCN and reconstituted derivatives even when the signals are buried under the intense diamagnetic envelope. This provides the value of the mean and spread of heme methyl hyperfine shifts that have been suggested to serve as a probe for the degree of imidazolate character of the proximal histidine[23] and the nature and magnitude of in-plane rhombic distortion in the protein, respectively.[16,24,25]

For the metcyano complex of proteins highly homologous to sperm whale Mb, the magnitude of inter-methyl NOEs appears characteristic and should aid significantly in allowing rapid determination of the heme orientation without resorting to isotope labels: the most shifted methyl does (8-CH_3) or does not (5-CH_3) show a small ($\sim$-1%) NOE to another heme methyl (1-CH_3). The method can be extended to any low-spin ferric hemoprotein, provided one amino acid in dipolar contact with one heme methyl can be located. Thus, the detection of NOEs between specific assigned methyl groups and the assigned protons of Ile-99 (FG5) allows the determination of the orientation of the heme in the heme cavity with respect to the α,γ meso axis.

In the two hemin analogues that have either the 6- or 7-propionate replaced by a methyl, a single heme orientation dominates ($\geq$90%) at equilibrium, and that orientation has the vinyl groups in the identical position as in the native protein.[2,10,17] Thus, we conclude that preferences in forming salt bridges between propionates and amino acid side chains (6-propionate with His-FG3 and 7-propionate with Arg-CD3) are much less important than preferences for vinyl-protein contacts in determining the equilibrium heme orientation in sperm whale myoglobin.

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Coenzyme Stereospecificity of Alcohol/Polyol Dehydrogenases: Conservation of Protein Types vs. Functional Constraints

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Abstract: A hypothesis is presented that allows prediction of the stereospecificity of alcohol and polyol dehydrogenases. The zinc-containing family with higher subunit molecular weight is *pro-R*; the family without zinc and lower subunit molecular weight is *pro-S* specific with respect to the coenzyme. New data for ethanol dehydrogenase from *Leuconostoc mesenteroides* and for glycerol dehydrogenase from *Bacillus megaterium* support this classification. Criteria for selecting systems suitable to contribute to the recent debate about functional constraints and conservation of coenzyme stereospecificity are suggested.

For the pyridine coenzyme NAD(P)-dependent dehydrogenases so far investigated it has been observed that the syn conformation of enzyme-bound nicotinamide leads to *pro-S* and the anti conformation to *pro-R* specificity of hydrogen transfer from NAD(P)H to carbonyl compounds.[1] Benner et al.[2,3] postulated that the energy difference between these conformers was used in biological evolution to put a functional constraint on enzymes catalyzing these redox reactions involving NAD(P). The function-based theory demands that reactive carbonyl compounds ($-\log_{K_{eq}} > 11.2$) are reduced by the *pro-R* and the less reactive ones ($-\log_{K_{eq}} < 11.2$) by the *pro-S* hydrogen of NAD(P)H. Controversial discussions have been published[4,5a,b] about the predictive potential of this new theory as compared to that of historical models, which are based on conservation of coenzyme stereospecificity by conservation of structural integrity of the proteins. According to Benner et al.,[6] the opposite stereospecificity of *Drosophila* and yeast alcohol dehydrogenase strongly supports the function-based theory, since these enzymes catalyze a reaction with low selective pressure. The equilibrium constant here lies

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at the borderline between *pro-S* and *pro-R* specificity.

On the bases of structural, functional, and genetic properties, which in our view have not been fully appreciated until now, we first want to demonstrate that *Drosophila* ADH is not a suitable example for testing such a hypothesis. We also want to explore additional criteria for the selection of enzymes that could reveal the proposed functional constraint. This will be discussed in detail for alcohol dehydrogenase from *Leuconostoc mesenteroides*. As a result of our investigations we found that the *pro-R* and *pro-S* stereospecificities found for yeast and *Drosophila* ADH are compatible with Bentley's first rule,[7] demanding that the stereospecificity of a particular reaction is fixed and does not depend on the source of the enzyme, since these alcohol dehydrogenases belong to two different enzyme classes.

Benner[6] based the degree of relationship between *Drosophila* and yeast ADH upon the existence of the β-sheet pattern, which was found in alignment studies and secondary-structure predictions.[8,9] However, the conclusion on a common ancestor between *Drosophila* and yeast ADH at a stage where ethanol oxidation could exercise a positive selection pressure does not follow from these studies. The $\beta\alpha\beta$ pattern is typical for the coenzyme-binding domain of all NAD(P)-dependent dehydrogenases;[10,11] it represents the part of the enzyme where the ADP moiety is bound.[12] Therefore, it supports only weak arguments regarding evolutionary relationships between different dehydrogenases. No relationship exists between the catalytic domains of *Drosophila* and yeast ADH, and the coenzyme-binding domain is located at the N terminus in *Drosophila* and at the C terminus in yeast ADH. On the basis of secondary-structure prediction, Thatcher[9] concluded that yeast and *Drosophila* ADH are totally unrelated, whereas Jörnvall et al.[8] suggested a common building block.

Our main reservation with respect to Benner's conclusions from the *Drosophila* ADH studies is the fact that this enzyme has not evolved for optimal catalysis of ethanol oxidation, which is one prerequisite of Benner's theory.

The turnover number per catalytic center (k_{cat}) is 1.4 s^{-1} for *Drosophila* ADH-S and 2.8 s^{-1} for the alleloenzyme *Drosophila* ADH-F[13] compared to $k_{cat} > 100$ s^{-1} [14] for yeast ADH. Turnover numbers for secondary alcohols decrease strongly for yeast ADH[14] and increase about threefold for *Drosophila* ADH.[15] The k_{cat}/K_m ratio for secondary alcohols as a measure of catalytic efficiency is up to 50 times higher for *Drosophila* ADH and about 400 times lower for yeast ADH compared to k_{cat}/K_m for ethanol. Benner et al.[6] cite several biological studies that in their view prove that ethanol is the only natural substrate for *Drosophila* ADH. These studies indicate that this enzyme is responsible for detoxification of ethanol, yet it is clear that it is not essential for energy production since flies without this ADH can also survive.[16] According to genetic features, enzymes may be divided into two groups.[17] One shows high substrate specificity and low heterozygocity, which may be compatible with high evolutionary pressure; enzymes engaged in energy metabolism belong to this group. The other group is "substrate-nonspecific" and is characterized by a high heterozygocity. Alcohol dehydrogenases from different species of *Drosophila* have been classified as belonging to the latter group.[18]

In conclusion, *Drosophila* ADH shows the typical behavior of a multiple-substrate-specific enzyme: high heterozygocity, which may be compatible with low functional constraints, broad substrate specificity, and low turnover numbers.

Results and Discussion

Alcohol Dehydrogenase from *L. mesenteroides*. *L. mesenteroides* ferments glucose to lactic acid, ethanol, and CO_2. One step in this catabolic process is the reduction of acetaldehyde, which is catalyzed by an ethanol dehydrogenase.[19] This enzyme represents a good candidate for testing any violations of conservation rules for the following reasons:

(i) The enzyme catalyzes a reaction that is essential for energy production.

(ii) Substrate binding sites are similar in yeast and *L. mesenteroides* ADH,[20] the turnover numbers for ethanol are in the same range, and substrate specificity is relatively narrow.[19,21]

(iii) The coenzyme binding sites show differences. Yeast ADH uses exclusively NAD^+ as coenzyme, while *Leuconostoc* ADH can operate with NAD^+ or $NADP^+$.

(iv) In dual-nucleotide-dependent dehydrogenases both syn and anti conformers of bound coenzyme have been detected, especially in cases with weakly bound coenzyme.[22] The dissociation constant of NAD^+ ($K_{E\cdot NAD^+} = 0.9 \times 10^{-3}$ M) is one of the highest found in ethanol dehydrogenases.[21]

(v) Differences between NADH and NADPH binding have also been shown by fluorimetric measurements.[20]

In view of these facts a violation could be possible of Bentley's second rule,[7] which requires the same stereospecificity for enzymes that can react with both NAD^+ or $NADP^+$. The NADP-dependent reaction was reported to be *pro-R* specific.[23] Only the NAD-dependent reaction is important for this discussion since, for the production of ethanol in growing cells of *L. mesenteroides*, NADH is the source of hydrogen.[24] No violation of conservation rules was found: we observed *pro-R* specificity for hydrogen transfer from ethanol to both NAD^+ and $NADP^+$.

Glycerol 2-Dehydrogenases. Glycerol is used by many microorganisms for energy production. The equilibrium constant of the reaction is close to the borderline of Benner's correlation.

pro-R specificity was reported for a poorly characterized glycerol dehydrogenase from *Aerobacter aerogenes*[5] and *pro-S* specificity for a dihydroxyacetone reductase from *Mucor javanicus*.[25] Dihydroxyacetone was found in the mycelium of this organism,[26] but the enzyme also shows high reactivity with decalones, for example.

Recently, glycerol dehydrogenase from *Bacillus megaterium* was reported to be a zinc enzyme.[27] It is related to yeast ADH

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Table I. Correlation between Coenzyme Stereospecificity and Protein Properties for NAD(P)-Dependent Alcohol/Polyol Dehydrogenases

enzyme	EC	subunit MW	active-site zinc	coenzyme stereochem[a]
alcohol dehydrogenase (yeast)	1.1.1.1	38 000[b]	+[b]	*pro-R*[c]
alcohol dehydrogenase (horse liver)	1.1.1.1	42 000[b]	+[b]	*pro-R*[d]
alcohol dehydrogenase (*L. mesenteroides*)	1.1.1.2	33 000[e]	+[e]	*pro-R*
alcohol dehydrogenase (*Drosophila melanogaster*)	1.1.1.1	27 000[f]	–[f]	*pro-S*[g]
glycerol 2-dehydrogenase (*B. megaterium*)	1.1.1.6	36 000[h]	+[h]	*pro-R*
dihydroxyacetone reductase (glycerol 2-dehydrogenase) (*M. javanicus*)	1.1.1.156	28 000[i]	(–)[k]	*pro-S*[i]
sorbitol dehydrogenase (sheep liver)	1.1.1.14	35 000–40 000[l]	+[m]	*pro-R*[n]
ribitol dehydrogenase (*Kelbsiella pneumoniae*[r])	1.1.1.56	24 000[o]	–[p]	*pro-S*[q]

[a] The *pro-R* hydrogen (H_R) is often called H_A, the *pro-S* hydrogen (H_S) H_B. [b] Reference 28. [c] Reference 29. [d] References 30 and 31. [e] Reference 20. [f] References 9 and 32. [g] Reference 6. [h] Reference 27. [i] Reference 25. [k] The requirement for zinc ions is not known.[25,26] [l] Reference 33. [m] Reference 34. [n] Reference 35. [o] Reference 36. [p] Reference 37. [q] Reference 38. [r] This organism is classified as *Aerobacter aerogenes* in the older literature; now it is named either *Klebsiella pneumoniae* or *Klebsiella aerogenes*.

and sorbitol dehydrogenase, which both contain zinc bound to cysteine in the active site. We find *pro-R* specificity for this glycerol dehydrogenase. This example indicates a novel possibility for correlating coenzyme stereospecificity with a similar active-site structure even for enzymes that react with different substrates.

"Long" Forms of Ethanol and Polyol Dehydrogenases as *pro-R* and "Short" Forms as *pro-S* Specific—A Postulate. On the basis of sequence homology studies of coenzyme binding and catalytic domains, Jörnvall et al.[8] divided ethanol and polyol dehydrogenases into two protein types: short and long forms with molecular subunit weights of about 25 000 and 40 000. We postulate that the long forms, which contain zinc in the catalytic site, are *pro-R* and the short forms without zinc are *pro-S* specific. This is demonstrated in Table I.

Whereas the existence of two classes of alcohol/polyol dehydrogenases showing different coenzyme stereospecificity seems to be well established, it remains an open question why such divisions have not been observed in other dehydrogenases, e.g. lactate or malate dehydrogenases. According to Benner, the energy difference between *pro-R* and *pro-S* specificities is so large that only one pathway is possible. Another explanation, however, could be as follows: Lactate and malate dehydrogenases show rather narrow substrate specificity. Alcohol and polyol dehydrogenases can have more narrow or very broad substrate specificity. The presence of both *pro-R* and *pro-S* coenzyme stereospecificities could then be due to different biological functions of these enzymes.

Our correlation could be tested only with a few examples of a huge family. Jörnvall et at.[37] included glucose dehydrogenase in their studies. It will be interesting to see whether sugar dehydrogenases fit into the proposed correlation. Primary structures for alignment studies are available for only a few enzymes, but as shown for glycerol dehydrogenases and alcohol dehydrogenase from *L. mesenteroides*, a prediction about coenzyme stereospecificity can be made by using simple criteria like subunit molecular weight and zinc content. More distantly related alcohol dehydrogenases are known with higher molecular weight, probably lacking zinc. A zinc-dependent mechanism implies a distinctly different catalytic pathway as compared to a chemical pathway without metal. Therefore, a different design of the active site is necessary. Whether this leads to different coenzyme stereospecificity (which could mean that a zinc-mediated mechanism is a final arbiter for *pro-R* and a mechanism without zinc for *pro-S* specificity) has to be examined for a broader range of alcohol/polyol dehydrogenases.

Conclusions. The systems selected show that within one structurally defined enzyme class the coenzyme stereospecificity is preserved. This supports historical models but cannot exclude functional constraints, as proposed by Benner's intriguing hypothesis. The new correlation allows prediction of coenzyme stereospecificity, which can be predicted neither by Benner's theory nor by Bentley's generalizations.

Experimental Section

Materials. The dinucleotides NAD^+ and $NADP^+$ (grade I), alcohol dehydrogenase from yeast (EC 1.1.1.1) and from *L. mesenteroides* (EC 1.1.1.2) were purchased from Boehringer, Mannheim. Glycerol dehydrogenase from *B. megaterium* (EC 1.1.1.6) was a gift from Dr. W. Brümmer. Ethanol-d_6 (99% deuterium), glycerol, and semicarbazide were obtained from Merck, Darmstadt. AG-MP-1 and Bio-Gel P-2 are available from Bio-Rad. Glucose 6-phosphate dehydrogenase from *L. mesenteroides* (EC 1.1.1.49) and dithioerythritol (DTE) were obtained from Sigma, and D-glucose-*1*-d_1 (96.1% deuterium) was from MSD Isotopes. All other chemicals were the purest commercially available. Double-distilled water was used throughout.

[4-^{2}H]NAD$^+$. [4-^{2}H]NAD^+ was obtained by oxidizing [4(*S*)-^{2}H]-NADH, which was prepared according to Viola et al.[39] After chromatography on a Bio-Gel P-2 column (2.5 × 50 cm; eluent 3 mM sodium pyrophosphate buffer, pH 8.8), acetaldehyde was added to the fractions containing [4(*S*)-^{2}H]NADH and the pH was adjusted to 7.2. The reaction was started with 50 mg of yeast ADH. After 10 min at 20 °C, the absorption at 340 nm decreased to 0. The enzyme was denatured with CCl_4 and centrifuged. The clear solution was applied to an AG-MP-1 column (2.2 × 17 cm, formate form), and the [4-^{2}H]NAD^+ was eluted with a linear gradient of 0–1 M formic acid and lyophilized. The concentration of [4-^{2}H]NAD^+ was determined in analogy to the described procedures.[40] The deuterium content was 96 ± 1% as found by comparing the integration of the proton resonance at the pyridine in the 4-position with that at the 6-position.

Instruments. ^{1}H NMR spectra were recorded on a Bruker AM 400/ASPECT-3000 system.

Stereoselectivity of Hydrogen Transfer Catalyzed by Alcohol Dehydrogenase from *L. mesenteroides*. The enzyme was assayed as described.[20] The reaction mixture (20 mL) contained 50 mM sodium pyrophosphate/hydrochloride, pH 8.8, 1 mM DTE, 22 mM semicarbazide, 150 mM ethanol-d_6, ca. 50 units of enzyme, and 5 mM NAD^+ or 5 mM $NADP^+$. After 90 min at 20 °C, the enzyme was removed as described above. The reduced coenzymes were purified by chromatography on an AG-MP-1 column (2.2 × 17 cm, Cl^- form), using a gradient of 0–0.5 M LiCl, pH 9.5. Salt was removed from the coenzyme-containing fractions with Bio-Gel P-2 (column, 2.5 × 50 cm; eluent; 3 mM sodium pyrophosphate, pH 8.8). The coenzymes were lyophilized and dissolved in D_2O (99.8% deuterium); this was repeated twice. With NAD^+ 4 ± 1% [4(*R*)-^{1}H]- and 96 ± 1% [4(*S*)-^{1}H]NADH were found, and with $NADP^+$ 7 ± 1% [4(*R*)-^{1}H]- and 93 ± 1% [4(*S*)-^{1}H]NADPH were found, by using the ^{1}H NMR technique devised by Arnold et al.[41]

Stereoselectivity of Hydrogen Transfer Catalyzed by Glycerol Dehydrogenase from *B. megaterium*. The enzyme was dialyzed and reactivated as described.[27] The reaction mixture contained 3 M glycerol, 3 mM [4-^{2}H]NAD^+ (96 ± 1% deuterium), 1 mM DTE, and ca. 50 units of enzyme in 12 mL of sodium pyrophosphate buffer (220 mM, pH 8.8).

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After 30 min at 20 °C, 81% of the coenzyme was reduced. The enzyme was removed, the coenzyme was purified, and the samples for ^1H NMR measurement were prepared as described with *Leuconostoc* ADH. We found 84 ± 1% [4(*R*)-^1H]- and 16 ± 1% [4(*S*)-^1H]NADH. Four percent of the nonspecific hydrogen transfer is due to the impurity of [4-^2H]-NAD^+; the rest possibly stems from nonenzymatic hydrogen transfer between NADH and NAD.[42]

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Registry No. NAD^+, 53-84-9; $NADP^+$, 53-59-8; [4-^2H]NAD^+, 60797-91-3; [4(*S*)-^2H]NADH, 10021-11-1; EC 1.1.1.1, 9031-72-5; EC 1.1.1.2, 9028-12-0; EC 1.1.1.6, 9028-14-2; EC 1.1.1.156, 39342-20-6; EC 1.1.1.14, 9028-21-1; EC 1.1.1.56, 9014-23-7; dehydrogenase, 9035-82-9.

Vibrational Circular Dichroism of Polypeptides. 8. Poly(lysine) Conformations as a Function of pH in Aqueous Solution†

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Abstract: The conformational transitions of poly(L-lysine) in D_2O solution over the range of pH 7-12 have been studied by using vibrational circular dichroism (VCD). Results in the amide I region show that gradual changes in VCD sign patterns qualitatively reflect the expected transitions from random-coil through right-handed α-helical to antiparallel β-sheet structures. The data indicate that, under our high-concentration measurement conditions, intermediate structures may occur during the pH titration and that at high pH the α-helix is conformationally unstable with respect to β-sheet formation. Additional data on the poly(lysine) conformation in methanol-water solution are presented to illustrate the VCD of the stable α-helix and to study the coil-helix transition in this solvent. This is the first report of solution-phase β-sheet VCD and the first report of VCD for all three major secondary structural types in the same polypeptide. Additionally, these data conclusively demonstrate that VCD is a viable technique for measurements on aqueous solutions and that information regarding secondary structure is available from such measurements.

Recently, we and others have demonstrated that vibrational circular dichroism (VCD) can be used to differentiate among the characteristic secondary structures of polypeptides.[1-10] These studies have shown that protonated α-helical structures yield bisignate amide I and amide A bands[1,2] and a monosignate amide II band[3] whose signs are dependent on the screw sense of the helix and not on the chirality of the α-carbon atom. For right-handed helices, the amide I line shape is modified to a three-peak pattern upon deuteration.[3] Other structural types such as the random coil[6] and 3_{10}-helix[7] have been shown to give solution-phase VCD that is distinguishable from that of the α-helix. In films, β-sheet structures gave frequency-shifted and lower amplitude VCD as compared to α-helices.[4] Up to this time, solution-phase polypeptide β-sheet VCD has not been reported. Polypeptide VCD in aqueous solution, with reasonable signal-to-noise ratio (S/N), has also proven to be elusive. This paper contains the first examples of both.

Poly(lysine) can provide a good test of the characteristic nature of the above VCD measurements in that it is reported to undergo transitions from random-coil through α-helix to β-sheet structures with variation of pH and temperature.[11-17] This variety of structures is facilitated by its polyionic nature due to the lysine side chain. These three structural types have been used by Greenfield and Fasman[16] and others[18-20] as the basis for methods of deconvoluting the electronic CD of globular proteins into component parts. Such procedures have subsequently led to assignment of the fraction of various secondary structural units present in a variety of proteins.

Here, we will present the VCD of these same structural types for poly(lysine), show that it is distinctive for each, and suggest that it could provide the basis of a new method for conformational analysis of polypeptides and proteins. Second, we will demonstrate that the structural transitions proposed for this system are not as simple as was previously thought, that the α-helical state is

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