Table II

entry	irradiation time, min	sensitizer	% product
1	30	none	58.6
2	60	none	>80.3
3	30	benzophenone	43.6
4	60	benzophenone	75.1
5	30	xanthone	85.8
6	60	xanthone	>90.0

Table III

entry	irradn time, min	% **24a**	equiv of piperylene
1	30	43.8	0
2	30	42.9	1
3	30	42.4	10
4	60	16.1	10
5	30	54.6	100
6	60	25.4	100

(0.22 g, 1.0 mmol). The now tan-colored solution was stirred at room temperature for 2 h, and then ethyl acetate was added. The organic phase was washed with water and brine. After the mixture was dried over magnesium sulfate, the solvent was removed under reduced pressure to give a black solid (53 mg, 94%). Chromatography on silica gel (dichloromethane–ethyl acetate, 9:1) provided a colorless solid (25 mg, 45%) identical with the previously isolated hydrindane **32**. The crude cyclobutene **31** (25 mg) was dissolved in dichloromethane (2 mL) and cooled to 0 °C, *m*-chloroperbenzoic acid (17 mg, 1.0 equiv) was added, and the solution was stirred for 1.5 h. Additional dichloromethane was then added, the solution was washed with saturated sodium bicarbonate solution and then brine. After drying over magnesium sulfate, the solvent was removed under reduced pressure to give **33** as a colorless solid (23 mg, 86%) identical with that previously isolated.

Triplet-State Sensitization of the Conversion of 24a to 25a. A solution of **24a** (10 mg) and benzophenone (22 mg) in benzene (2 mL) was prepared. Benzophenone absorbed 95% of the light of wavelength 366 nm while the remainder of this light was absorbed by **24a**. Similarly, a solution of **24a** (10 mg) and xanthone (6.6 mg) in benzene (2 mL) was prepared. Xanthone absorbed 39% of the 366-nm light and 86% of the 357-nm light. A control sample containing **24a** (10 mg) in benzene (2 mL) also was prepared. All samples were purged with dry nitrogen for 15 min prior to irradiation and then were simultaneously irradiated by a 400-W Hanovia medium-pressure mercury arc lamp fitted with a uranyl glass filter sleeve. Irradiations were performed for 30 and 60 min, and analyses were carried out with a Hewlett-Packard HP-5710A gas chromatograph fitted with a 16 ft × $^1/_8$ in. stainless steel column. The column was packed with 80–100 mesh Chromosorb G-HP coated with 0.5% QF-1. A flame-ionization detector and a HP-3380A integrator were used for the quantitative analyses.

Attempted Quenching of the Conversion of 24a to 25a. Samples were prepared by dissolving **24a** (10 mg) and 4, 40, or 400 μL (1, 10, 100 equiv) of piperylene in benzene to a total volume of 2.5 mL. A control sample was prepared by dissolving **24a** (10 mg) in benzene (2.5 mL). All samples were purged with dry nitrogen for 15 min prior to irradiation and then were simultaneously irradiated by a 400-W Hanovia medium-pressure mercury arc lamp fitted with the uranyl glass filter sleeve for 30 or 60 min. The samples were analyzed by use of the gas chromatograph.

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Supplementary Material Available: Tables of crystal structure data, atomic coordinates, bond lengths, bond angles, anisotropic parameters, and hydrogen atom coordinates for **12a** and **16a** (12 pages). Ordering information is given on any current masthead page.

Stereochemical Profile of the Dehydrogenases of *Drosophila melanogaster*

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Abstract: A "stereochemical profile" has been experimentally constructed for dehydrogenases from *Drosophila melanogaster*, an organism known to contain an enzyme with an "unusual" stereospecificity. Three of the enzymes examined (malate dehydrogenase, isocitrate dehydrogenase, malic enzyme) catalyze the transfer of the *pro-R* (A) hydrogen from NAD(P)H. Three other enzymes (glucose 6-phosphate dehydrogenase, alcohol dehydrogenase, glycerol 3-phosphate dehydrogenase) catalyze the transfer of the *pro-S* (B) hydrogen from NAD(P)H. The stereospecificity of alcohol dehydrogenase is notable because it is the opposite of that of alcohol dehydrogenases from yeast and mammals, with respect both to cofactor and to the enantiotopic hydrogens on ethanol. These results, together with published data, suggest a general working hypothesis regarding natural selection and the cryptic stereospecificity of enzymes. Natural selection will not distinguish between "locally enantiomeric" transition states; enzymes catalyzing analogous reactions via both transition states should be found in nature. In contrast, natural selection in general will distinguish between enzymes catalyzing analogous reactions via "locally diastereomeric" transition states; in general, only a single diastereomeric transition state should be found in naturally occurring enzymes.

Interest in the stereospecificity of dehydrogenases dependent on nicotinamide cofactors has undergone a renaissance since the proposal of several new functional, structural, and historical models explaining what previously was regarded as a nonfunctional behavior.[1-4]

Distinguishing between these models is challenging, as it requires an assignment of the relative importance of natural selection, conservation, and neutral drift in the recent evolution of modern proteins.[5-7] Nevertheless, the distinction is important, as the

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interpretation of all bioorganic data is affected by presumptions regarding selection and drift at the molecular level. Further, if the distinction is made for a few macromolecular behaviors, these may be useful to order the selectability of other behaviors. Arguments that stereospecificity in dehydrogenases, with a presumed impact of only 1–2 kcal/mol on the kinetic behavior of an enzyme, is a selected trait suggest that other traits with larger impacts are also selected. Conversely, arguments that stereospecificity in dehydrogenases is not selected suggest that traits with smaller impacts on enzymatic behavior are also not selected.[8]

A research paradigm making this distinction must consider the evolutionary status of enzymes chosen as examples. If stereochemical data are sought to distinguish between functional and nonfunctional models for stereospecificity, comparing dehydrogenases from two closely homologous organisms is generally of little value, as both models predict that the stereospecificities will be the same. Similarly, enzymes with analogous functions from organisms as evolutionarily distant as possible serve as the best tests of models that assume that stereospecificity is highly conserved for functional reasons. In such enzymes, it is most likely that sufficient time has passed for stereospecificity to have diverged if it is not functionally constrained.

To encourage the collection of stereochemical data in a form that is likely to assist in the development of functional and historical models, we suggest here a new concept, that of a "stereochemical profile" of an organism. An organism is chosen because stereochemical information suggests that it is evolutionarily distant from other organisms containing enzymes with known stereospecificities, and perhaps sufficiently distant that the stereospecificity of its enzymes would not be conserved for nonfunctional reasons. For example, a suitable organism might have one enzyme whose stereospecificity is opposite that of the stereospecificity of analogous enzymes from other well-studied organisms. The organism is then examined to determine which other stereochemically interesting enzymes it contains, and the stereospecificities of these enzymes are determined.

We have chosen *Drosophila melanogaster* to illustrate this approach, because the alcohol dehydrogenase (Adh) from *Drosophila* was recently shown to catalyze the transfer of the *pro-S* hydrogen of NADH,[5] a stereospecificity opposite that of the alcohol dehydrogenases from mammals and yeast. The fact that *Drosophila* is sufficiently distant from these other organisms to have an alcohol dehydrogenase with opposite stereospecificity suggests that *other* dehydrogenases from *Drosophila* might also have divergent stereospecificities if stereospecificity is not a selected trait.

We report here the stereoselectivities of six dehydrogenases from *D. melanogaster*, together with the stereospecificity of the alcohol dehydrogenase from *Drosophila* with respect to the two enantiomeric hydrogens on ethanol.

Experimental Section

Preparation of Tritiated Coenzymes. [4-^{3}H]NAD$^+$, [^{3}H]NADP$^+$, [4(*R*)-^{3}H]NADH, and [4(*S*)-^{3}H]NADPH [4(*S*)-^{3}H]NADH were prepared by enzymatic reduction of NAD$^+$ (0.2 μmol) with [1-^{3}H]glucose 6-phosphate catalyzed by glucose 6-phosphate dehydrogenase (2 units) from *Leuconostoc mesenteroides* in Tris buffer (2 mL, 0.1 M, pH 8.0). The [1-^{3}H]glucose 6-phosphate was prepared in situ with hexokinase (5 units) from [1-^{3}H]glucose (0.04 μmol) and ATP (0.5 μmol). The reaction was monitored spectrophotometrically (340 nm). [4-^{3}H]NAD$^+$ was prepared by oxidation of the [4(*S*)-^{3}H]NADH with acetaldehyde (10 μL, distilled) catalyzed by yeast alcohol dehydrogenase (5 units). [4(*S*)-^{3}H]NADPH was similarly prepared by the reduction of NADP$^+$ by [1-^{3}H]glucose 6-phosphate catalyzed by glucose 6-phosphate dehydrogenase. The [4(*S*)-^{3}H]NADPH was reoxidized with dihydrofolate (0.2 μmol) catalyzed by dihydrofolate reductase (1 unit) to give [4-^{3}H]NADP$^+$. [4(*R*)-^{3}H]NADH and [4(*R*)-^{3}H]NADPH were prepared by the reduction of [4-^{3}H]NAD$^+$ and [^{3}H]NADP$^+$ with unlabeled glucose 6-phosphate in the presence of glucose 6-phosphate dehydrogenase. Labeled nicotinamide cofactors were purified by DEAE-cellulose chromatography (HCO_3^- form, elution with a gradient of a 0–1 M NH_4HCO_3) or by HPLC. The fractions with satisfactory ultraviolet spectra were combined and lyophilized.

Malate Dehydrogenase. Headless files (5.4 g) were suspended in buffer (K_2HPO_4, 50 mM, pH 7.0, containing 1 mM mercaptoethanol and 1 mM EDTA, hereafter referred to as "phosphate buffer") and disrupted in a Biospec glass bead mill (2 × 1 min). The mixture was allowed to settle and the supernatant decanted; the beads were washed with phosphate buffer. The combined supernatant and washings were then centrifuged and clarified by filtration through glass wool.

To the filtrate (total volume 34 mL) was added 4.8 mL of a 2% solution of protamine sulfate (neutralized in phosphate buffer). The mixture was stirred gently for 30 min and then centrifuged (31000*g*, 15 min). The supernatant was fractionated with NH_4SO_4. The activity was found to precipitate between 45% and 70% saturation. The pellet was redissolved in 1.2 mL of buffer (Tris-HCl, 50 mM, pH 8.3, containing 1 mM mercaptoethanol and 1 mM EDTA, hereafter referred to as "Tris buffer") and dialyzed overnight against 2 L of Tris buffer.

The dialysate was centrifuged at 13000*g* (15 min) to remove a brown precipitate, and the supernatant was chromatographed on DEAE-Sephadex (0.7 × 15 cm) equilibrated in Tris buffer. The activity eluted in the first 15 mL, which was pooled and concentrated to 1.5 mL in a Amicon ultrafiltration cell (cutoff M_r 10000).

To determine the stereospecificity of malate dehydrogenase, [4(*S*)-^{3}H]NADH (0.1 mM) was oxidized by the malate dehydrogenase preparation (1 μL) in phosphate buffer containing oxaloacetate (2 mM). Products of the reaction were isolated by thin-layer chromatography on PEI-cellulose eluted with 0.25 M lithium chloride. The corresponding fractions were collected, extracted (1 N NH_4OH), and counted on a Beckmann scintillation counter.

A solid derivative of the product malate was prepared and recrystallized to constant specific activity. Carrier L-malate (0.2 g, 1.5 mmol in 5 mL of water) was added to the ultrafiltered reaction mixture containing enzymatically formed malate; the mixture was diluted with 6 mL of ethanol containing phenacyl bromide (0.67 g) and refluxed for 48 h. The phenacyl ester precipitated after the addition of water and cooling and was recovered by filtration, dried, and recrystallized to constant specific activity from benzene and low-boiling petroleum ether.

Malic Enzyme. Malic enzyme was purified to apparent homogeneity following a literature procedure.[9] *Drosophila* (5 g in 25 mL of 0.1 M Tris buffer, pH 7.6, containing 5 mM EDTA and 1 mM mercaptoethanol) were disrupted in a Biospec bead beater (2 × 1 min); the extract was decanted, centrifuged, and clarified as above and then treated with protamine sulfate (2% solution in Tris buffer, 1.4 mL/10 mL of supernatant); the mixture was stirred (30 min) and centrifuged (30000*g*, 30 min). The supernatant was fractionated by treatment with NH_4SO_4. Malic enzyme precipitates between 45% and 60% saturation. The pellet was redissolved in 100 mM Tris buffer (0.5 mL) and dialyzed twice against 1 L of Tris buffer.

This extract was further purified by affinity chromatography on 2′,5′-ADP–Sepharose 4B (2 g) preequilibrated with Tris buffer. Malic enzyme was eluted with a linear gradient (150 mL) of NADP$^+$ (0–0.6 mM) in Tris buffer and concentrated by ultrafiltration. The final volume was 485 μL and contained 4.8 units with a specific activity of 16.7 (purification 128-fold). The protein appeared homogeneous by SDS chromatography.

The stereospecificity of the malic enzyme activity was determined by incubating [4-^{3}H]NADP$^+$ (0.25 mM, 10^6 cpm) with malate (0.3 mM) and enzyme in Tris buffer (1 mL) containing manganese chloride (10 mM). The reaction was monitored spectroscopically at 340 nm. When the increase in absorption leveled off, the [4(*S*)-^{3}H]NADPH was oxidized in two separate batches.

In the first, the *pro-S* hydrogen of the NADPH was removed by incubation with glutamate dehydrogenase (bovine, 2 units) in buffer containing ammonium acetate (120 mM) and α-ketoglutarate (1 mM). The enzyme was removed by passing the solution through a Centricon 10 membrane, and the products (glutamate and NADP$^+$) were separated by silica thin-layer chromatography (eluant, 1-butanol/acetic acid/water 4:3:1), extracted, and counted.

In the second, the *pro-R* hydrogen was removed by incubation with dihydrofolate reductase (0.1 unit) in buffer containing dihydrofolate (0.5 mM) and mercaptoethanol. The enzymes were removed by ultrafiltration, and the products (tetrahydrofolate and NADP$^+$) were separated by thin-layer chromatography as above and counted.

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Isocitrate Dehydrogenase. An extract from flies prepared as for malic enzyme was treated with protamine sulfate as described above. This extract was directly used to determine the sterospecificity of the isocitrate dehydrogenase.

$NADP^+$ (0.27 mM, 2.0×10^6 counts/min) was reduced by extract (1.2 milliunits) in imidazole buffer (90 mM, pH 8.0, 1 mL) containing magnesium chloride (5 mM) and D-isocitrate (0.3 mM). The reaction was monitored spectrophotometrically. When it was complete, the reaction mixture was divided into two portions. In one, the *pro-S* hydrogen was removed from NADH by oxidation by 2-ketoglutarate and ammonia in the presence of glutamate dehydrogenase (bovine). The reaction products were separated either by thin-layer chromatography, as described above, or by cation-exchange chromatography on a Dowex 50 W-X2 resin. $NADP^+$ was eluted with water, the glutamate was eluted with 1 N NH_4OH.

In the other, [4-H^3]NADPH was isolated by HPLC (LKB-TSK DEAE-5-PW weak anion-exchange column, 7.5 × 75 mm), using a linear gradient of sodium chloride (0–500 mM) in Tris buffer (50 mM, pH 8.0). The *pro-R* hydrogen of the isolated labeled NADPH was removed by oxidation by dihydrofolate reductase (1 unit) in Tris buffer containing mercaptoethanol and dihydrofolate (0.3 mM). The reaction was monitored at 340 nm. After the reaction had ceased, $NADP^+$ and tetrahydrofolate were isolated by ultrafiltration, separated, and counted.

Glucose 6-Phosphate Dehydrogenase. A clarified extract from 1.2 g of flies, prepared as described for malic enzyme, was treated with a 2% protamine sulfate solution as above and fractionated with NH_4SO_4. The glucose 6-phosphate dehydrogenase activity precipitated between 40% and 80% saturation. The pellet was redissolved in extraction buffer (0.3 mL) and stored at −70 °C.

Extract (40 μL) was incubated with $NADP^+$ (1.1 mM), [1-^{3}H]glucose (0.28 mM, 10 μCi), hexokinase (2 units), and ATP (0.8 mM) in triethanolamine buffer (1 mL, 0.1 M, pH 7.6) containing $MgCl_2$ (5 mM). In a parallel reaction, extract (40 μL) was incubated with [4-^{3}H]$NADP^+$ (0.3 mM, 2×10^6 cpm) and glucose 6-phosphate (0.33 mM) in 1 mL of triethanolamine buffer containing $MgCl_2$. The reactions were monitored spectrophotometrically (340 nm). After further reaction ceased, NADPH was isolated from aliquots (0.2 mL) of each reaction mixture by HPLC (LKB-TSK DEAE-5PW weak anion-exchange column), using a gradient of 0–0.5 M NaCl in Tris buffer (50 m, pH 8.0). Each specimen of [4-^{3}H]NADPH was incubated with glutamate dehydrogenase (2 units) and 2-ketoglutarate (0.5 mM) in triethanolamine buffer (100 mM, pH 7.6) containing ammonium acetate (120 mM). After the reaction had ceased, the reaction mixtures were ultrafiltered, and [4-^{3}H]NADP and glutamate were separated and counted as described above.

Glycerol 3-Phosphate Dehydrogenase.[10] The clarified extract from 1.5 g of flies homogenized in Na_2HPO_4 buffer (10 mM, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) was incubated for 25 min at 50 °C and centrifuged (20000*g*, 20 min) to remove denaturated protein. The extract was stored at 20 °C.

The crude extract was used directly to determine the stereospecificity of the glycerol 3-phosphate dehydrogenase activity. [4(*S*)-^{3}H]NADH (0.28 mM, 4×10^6 cpm) was incubated with extract (0.02 mL) in 1 mL of triethanolamine buffer (100 mM, pH 7.8) containing dihydroxyacetone phosphate (0.6 mM). After the reaction was complete, the reaction products were isolated by ultrafiltration, separated, and counted as described above. Parallel experiments were executed with [4(*R*)-^{3}H]NADH.

Alcohol Dehydrogenase. Alcohol dehydrogenase from *Drosophila* was prepared as previously described[5] and used to catalyze the reduction of acetaldehyde with [4(*S*)-^{3}H]NADH to yield tritiated ethanol, which was characterized as its naphthylurethane derivative. The labeled ethanol was recovered and reoxidized by NAD^+ in the presence of semicarbazide with alcohol dehydrogenase from yeast, known to transfer the *pro-R* hydrogen of ethanol. After the reaction was complete, the acetaldehyde semicarbazone was diluted with carrier, identified, isolated by thin-layer chromatography, and counted. The product NADH was also isolated by ion-exchange chromatography and counted.

Results

The results of the stereochemical experiments are shown in Table I. Enzymes that operate in major metabolic pathways, preferably pathways already known in *Drosophila*, were examined, as this increases the probability (but does not guarantee) that the enzymes examined are acting on a physiologically well-defined substrate. Some knowledge of the detailed biochemistry is especially useful in making judgements on this issue. For example, the metabolism in *Drosophila* is highly "aerobic", and the organism is expected (and found) to have high levels of enzymes involved in the Krebs cycle.

Table I

enzyme	cofactor	stereospecificity of dehydrogenase from *Drosophila*	stereospecificity of dehydrogenase from other organisms
alcohol dehydrogenase	NADH	*pro-S*	*pro-R*
glucose 6-phosphate dehydrogenase	NADPH	*pro-S*	*pro-S*
glycerol 3-phosphate dehydrogenase	NADH	*pro-S*	*pro-S*
malate dehydrogenase	NADH	*pro-R*	*pro-R*
malic enzyme	NADPH	*pro-R*	*pro-R*
isocitrate dehydrogenase	NADPH	*pro-R*	*pro-R*

Adh from *Drosophila* was found to catalyze the removal of the *pro-S* hydrogen at C-1 of ethanol; in contrast, Adh from yeast catalyzes the transfer of the *pro-R* hydrogen of ethanol.

Discussion

Two types of models may explain the stereospecificity of dehydrogenases dependent on nicotinamide cofactors.[5] Functional models assume that the particular stereospecificities that evolve under selective pressure are those best suited to help the host organism survive and reproduce. In contrast, historical models interpret stereospecificity in terms of events in the evolutionary history of the protein and deny a selectable function for stereospecificity. Historical models view stereospecificity as a neutral trait that either is drifting randomly as the structure of the protein diverges or is sufficiently tightly coupled to other functional behaviors in the protein that it reflects a conserved accident in the evolution of an ancestral protein.[5]

It is clear that stereospecificity in dehydrogenases is not drifting, at least not very rapidly. For example, lactate dehydrogenses from 15 organisms, including 4 D-lactate dehydrogenases and 11 L-lactate dehydrogenases, all transfer the *pro-R* hydrogen from NADH.[11] Likewise, malate dehydrogenases from plants, animals, archaebacteria, *Drosophila* (reported here), and eubacteria all transfer the *pro-R* hydrogen.[11,12] To explain these facts, historical models must assume that stereospecificity, although not functional itself, is closely tied to other functional behaviors in dehydrogenases and, as a consequence, is very highly conserved. Further, such historical models must assume that substrate specificity in dehydrogenases has not drifted, at least not over wide ranges. This latter assumption is necessary to exclude the evolution of a *pro-S*-specific malate dehydrogenase from a *pro-S*-specific enzyme originally acting on another substrate.

In *Drosophila*, the stereospecificities of five of the six dehydrogenase activities examined are identical with the stereospecificities of analogous dehydrogenases from other organisms. These enzymes conform to the general rule that enzymes from different organisms displaying the same catalytic activity have the same stereospecificity ("Bentley's first rule").[13]

In principle, Bentley's first rule is consistent *both* with functional models that assume that enzymes with particular stereospecificities are optimally suited to catalyze reactions on specific substrates *and* with historical models that assume that stereospecificity and substrate specificity in dehydrogenases are highly conserved and that enzymes from all organisms that act on the same substrate are homologous. However, the alcohol dehydrogenase (EC 1.1.1.1) from *Drosophila* does not have the same stereospecificity as the analogous enzymes from two other organisms. In yeast and horse, this enzyme transfers the *pro-R* hydrogen;[11] in *Drosophila*, this enzyme transfers the *pro-S* hydrogen.[5]

(10) Kiesel, D. W.; Bewely, G. C.; Miller, S. G.; Armstrong, F. B. *J. Biol. Chem.* **1980**, *255*, 4073.

(11) You, K.-S. *Methods Enzymol.* **1982**, *87*, 101–126.

(12) Goerisch, H.; Hartl, T.; Grussebueter, W.; Stezowski, J. J. *Biochem. J.* **1985**, *226*, 885.

(13) Bentley, R. *Mol. Asymmetry Biol.* **1970**, *2*, 1–89.

Historical models find this violation of Bentley's first rule problematical. Either these alcohol dehydrogenases are homologous or they are not. If they are not homologous, the historical model must become more complicated by presuming the existence of two ancestral alcohol dehydrogenases, one selecting the *pro-R* hydrogen, the other the *pro-S* hydrogen. If they are homologous, historical models must postulate that the stereoselectivity with respect to cofactor can be reversed in the divergent evolution of the two modern enzymes from a single ancestral enzyme.

The second ad hoc hypothesis largely destroys the explanatory powers of historical models in light of available facts. An historical model that assumes that stereospecificity is anything less than absolutely conserved cannot easily explain the fact that the stereospecificities of the other five dehydrogenases from *Drosophila* conform the Bentley's first rule; nor can it explain the identical stereospecificities of the other malate and lactate dehydrogenases mentioned above. This is the significance of the suggestion[14] that the dinucleotide binding domains of alcohol dehydrogenases from horse, yeast, and *Drosophila* are homologous. If this suggestion is correct, homologous enzymes have opposite stereospecificities, and a simple assumption that all malate dehydrogenases are homologous is no longer logically satisfactory to explain the fact that all malate dehydrogenases have the same stereospecificity.[15]

However, for the sake of this discussion, we shall consider the possibility that homology of the dinucleotide binding domains is not relevant to the issue of stereospecificity. For example, it could be argued that the catalytic domain of these dehydrogenases is the sole determinant of stereospecificity. Thus, while the *dinucleotide binding domains* of Adh from yeast and *Drosophila* might be homologous, as long as the catalytic domains are not homologous, one might argue that the different stereospecificities of the two dehydrogenases are not truly divergent.

This is a dialectical position rather than one firmly grounded in our understanding of the structure of these proteins.[16,17] Nevertheless, this argument rescues the historical view with a model that assumes that there are multiple ancestral catalytic domains for Adh and that domain swapping is involved in the evolution of these proteins with divergent stereospecificities.

This rescue has a cost. First, any mechanism that is invoked to explain stereochemical diversity in one class of dehydrogenases (here, ethanol dehydrogenases) is also, in principle, a mechanism for obtaining stereochemical diversity in all classes of dehydrogenases. Thus, the question remains, if multiple ancestral catalytic domains existed, if they were swapped with a common ancestral dinucleotide binding domain during the time since *Drosophila* diverged from other branches of the evolutionary tree, and if this process created alcohol dehydrogenases with different stereospecificities, why did a similar process not produce similarly divergent stereospecificities in the other five dehydrogenases in *Drosophila*?

Further, any set of stereochemical data can be accommodated by a historical model that assumes an arbitrary pedigree arbitrarily interrelating modern proteins with an arbitrarily large number of ancestral domains. However, a historical model formulated on such premises is uncontradictable; any experimental contradiction can be accommodated by postulating another ancestral swapping event involving yet another ancestral catalytic domain. That this approach to defending historical models undermines their value as working hypotheses has only slowly been appreciated by the biochemical community.[18]

As often as not, claims that historical models are predictive require knowledge of the amino acid sequence of the proteins in question; often the prediction is little more than the statement that the closer the sequence similarities, the higher the probability that two enzymes will have the same stereospecificity. This is not a particularly significant statement; it applies to properties of enzymes generally and is never in dispute. Further, in the absence of a detailed knowledge of the relationship between sequence and stereospecificity in a dehydrogenase, and given pairs of enzymes with opposite stereospecificities that are at least partially homologous,[5] even this prediction must be so ambiguous as to be uncontradictable.

It is worthy of note that the stereochemical diversity in alcohol dehydrogenases can be understood in terms of a functional model that correlates stereoselectivity with the redox potential (or thermodynamic stability) of the substrate that the enzyme has evolved to reduce.[1] The model assumes that enzymes optimally evolved to reduce unstable carbonyls have evolved to transfer the *pro-R* hydrogen, while enzymes optimally evolved to reduce stable carbonyls have evolved to transfer the *pro-S* hydrogen. Neither stereospecificity is strongly preferred in enzymes acting on carbonyl substrates with intermediary stability. "Intermediary stability" refers to an equilibrium constant (K_{eq} = [NADH][carbonyl][H^+]/[NAD^+][alcohol]) of $\sim 10^{-11}$ M. The divergent stereospecificities of ethanol dehydrogenases, with substrates with $K_{eq} = 8 \times 10^{-12}$ M, are consistent with this picture.

We can summarize stereochemical data for dehydrogenases, all apparently consistent with the functional model mentioned above, but that together cannot easily be accounted for by a predictive historical model.

(1) The dinucleotide binding domains of ethanol dehydrogenases from yeast and *D. melanogaster* appear homologous by sequence comparisons. The enzymes catalyze a redox reaction on substrates with intermediary redox potential, and the enzymes have opposite stereospecificities with respect to cofactor.[5] Similarly, the dinucleotide binding domains of glyceraldehyde 3-phosphate dehydrogenases and lactate dehydrogenases appear to be homologous, on the basis of comparisons of their crystal structures.[16] These enzymes also have opposite stereospecificities. These data rule out hypotheses that stereospecificity of dehydrogenases is absolutely conserved.

(2) Dehydrogenases acting on ethanol (horse liver alcohol dehydrogenase) and sorbitol are clearly homologous.[14] This and data from other pairs of dehydrogenases (e.g., glucose dehydrogenase and ribitol dehydrogenase) rule out hypotheses that the substrate specificity of dehydrogenases is absolutely (or even highly) conserved.

(3) In D- and L-lactate dehydrogenases, homology is uncertain. However, the enzymes catalyze a reaction with a K_{eq} in the "*pro-R* region" of the correlation, act on substrates with opposite chiralities, yet have identical stereospecificities with respect to cofactor. Either these enzymes are homologous or they are not. If they are, historical models must explain why stereospecificity with respect to *substrate* has diverged while stereospecificity with respect to *cofactor* cannot. If they are not, historical models must

(14) Jornvall, H.; Persson, M.; Jeffrey, J. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4226.

(15) This statement would not be weakened by the argument, most recently presented by Schneider-Bernloehr and her colleagues,[4] that the oxidation of ethanol is not the physiological function of Adh from *Drosophila*, even if this argument were true. If Adh from yeast and *Drosophila* are homologous, a homologous pair of enzymes has opposite stereospecificity; this statement alone weakens historical models that must assume extremely high conservation of stereospecificity to explain other data. As a logical point, concerns of physiological function are relevant to the defense and/or criticism of functional models, not to the defense and/or criticism of historical models. In any case, the physiological function of Adh in *Drosophila* is almost certainly the oxidation of ethanol, at least in the modern world. For a review of the large amount of biological literature supporting this conclusion, see: Reference 5.

(16) Rossmann, M. J.; Liljas, A.; Braenden, C.-I. *Enzymes, 3rd Ed. 2*, **1975**, *11*, 61.

(17) This is not obvious from the crystal structure of the horse Adh, where stereospecificity seems to be determined by interactions between the cofactor and *both* domains.

(18) Despite the weakened stated of the historical picture, some partial models might be imagined. For example, both Schneider-Bernloehr and You have followed the suggestion of Jornvall[14] in dividing dehydrogenases into classes. "Large" enzymes (M_r ca. 40 000) are assumed to catalyze the transfer of H_R of NADH and require zinc ion for catalysis, while "small" enzymes (M_r ca. 27 000) catalyze the transfer of H_S of NADH and do not require zinc ion for catalysis. This model is only partly serviceable. While it is true that all alcohol dehydrogenases requiring metal transer the *pro-R* hydrogen of NADH, not all H_R-specific enzymes contain metals,[31] not all small dehydrogenases catalyze the transfer of H_S,[32,33] not all large dehydrogenases require metal,[34] and not all enzymes transferring H_S are small.[35] If we assume that metal ion requirement, size, and stereospecificity are sufficiently conserved to be markers of pedigree, this can be explained only by assuming no fewer than five ancestral dehydrogenases.

postulate another pair of ancestral dehydrogenases, this time with the same stereospecificities purely by chance. The functional model is unconcerned with the pedigree of these enzymes. If they are not homologous, the common stereospecificity converged for functional reasons. If they are homologous, stereospecificity was conserved for functional reasons.

(4) Several sets of enzymes from widely divergent sources catalyzing redox reactions far from the break in the correlation share a common stereospecificity. These are now best exemplified by the malate dehydrogenases. Functional models again view these enzymes as examples of either convergent evolution for functional reasons or conservation of stereospecificity for functional reasons. However, these sets require that historical models assume that stereospecificity is nearly absolutely conserved.

(5) Kraut and his co-workers have uncovered a pair of dihydrofolate reductases that, on the basis of crystallographic data, are almost certainly not homologous,[19] but which nevertheless have the same stereospecificity. Although the reduction of an imine to an amine is not within the scope of the original functional model,[1] we believe that this is the first clear example of convergent evolution of stereospecificity in dehydrogenases, and it again supports functional models over historical models.

(6) HMG-CoA reductases from *Acholeplasma* and yeast have opposite stereospecificities.[20] Their evolutionary interrelatedness is unknown. However, either they are related or they are not. If they are, the assumption inherent in all historical models, that stereoselectivity with respect to cofactor is highly conserved, must be discarded. If they are not, the historical models must again propose yet more discrete evolutionary ancestors for these dehydogenases.

Of course, these arguments do not *prove* the functional model based on the redox potential of the natural substrate,[21] and this model could certainly be contradicted by experimental data yet to emerge. However, the model remains a productive stimulus for experimental test. This statement seems especially true in light of recent experimental work showing analogous stereochemical behavior in model systems,[22] computational work on the chemical basis of the functional model,[23] and crystallographic and spectroscopic work on the properties of dihydronicotinamides.[24]

Drosophila presents another interesting stereochemical result. Accepting the functional model's assumption that stereospecificity with respect to cofactor in alcohol dehydrogenase is fortuitiously neutral, we can ask whether the sterospecificity with respect to the two enantiomeric hydrogens at carbon 1 of ethanol is the same in both dehydrogenases. Here again, there is the possibility of both historical and functional arguments. If the two enzymes transfer the same hydrogen from ethanol, the two transition states are "locally diastereomeric".[25] If both are found in nature, this suggests that natural selection does not notice the energetic differences between these two transition states. In contrast, if the two enzymes transfer the opposite hydrogens from ethanol, the two transition states are "locally enantiomeric" (now ignoring the stereocenters in the ribose rings),[25] implying that the two possible diastereomeric transition states, with different energies, are distinguishable by natural selection.

In fact, Adh's from yeast and *Drosophila* catalyze the redox reaction via locally enantiomeric transition states (again ignoring stereocenters on the ribose rings). More specifically, the transition states that are preferred are the ones where the steric bulk of substrate and cofactor are matched to minimize steric repulsion in the transition state.[26]

Some 15 years agon, George et al.[27] attempted to explain the stereoselectivity of dehydrogenases with respect to nicotinamide cofactor by the assumption that enzymes "choose" between "diastereomeric transition states" to minimize sterically unfavorable interactions between substrate and cofactor. The authors attempted to test this assumption by examining the stereospecificity of two steroid dehydrogenases, one oxidizing a β-hydroxysteroid, the other oxidizing an α-hydroxysteroid. They predicted that the cofactor stereospecificities would be opposite. In fact, both enzymes transferred the *pro-S* hydrogen.

The functional model dicussed above suggests that the systems available to the authors were not satisfactory to examine their conjecture. The two steroids had different (noncryptic) chiralities, and requirements for metabolic coupling dictate the absolute stereospecificity with respect to the chirality of the steroid. Further, both hydroxysteroids had redox potentials in the "*pro-S* region" of the correlation, suggesting that the *pro-S* hydrogen of NADH would be transferred for functional reasons. Thus, the more subtle diastereomerism predicted by George et al.[27] is (according to the functional model) hidden by these stereochemical imperatives. This is not the case for ethanol dehydrogenases, where stereospecificity with respect to substrate is cryptic (i.e., requiring isotopic substitution for detection) and where the redox potential of the natural substrate is such that no strong imperative exists for the stereospecificity with respect to cofactor.

Of course, studies of just two alcohol dehydrogenases cannot form the basis of a statistically satisfactory argument. However, these results, together with our recent report of the apparent lack

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(21) It is logically insufficient to argue, as is frequently done, that the stereospecificity of modern dehydrogenases can be explained by a model where stereospecificity is highly conserved but substrate specificity is easily altered. Diverging substrate specificity provides a mechanism for creating stereochemical heterogeneity within a class of enzymes that is just as effective as direct divergence of stereospecificity. For example, it is a common occurrence for the role of enzymes that are deleted to be taken over by another enzyme following a short evolution.[36] If substrate specificity is freely diverging, enzymes with *pro-S* stereospecificity can take over the role (for example) of deleted malate dehydrogenases having *pro-R* stereospecificity, creating stereochemical diversity within the class of malate dehydrogenases. That this has not happened implies rather rigid constraints on the drift of *both* substrate *and* stereospecificity. Nevertheless, conservation of substrate specificity clearly is not the rule in dehydrogenases.[14] Further, a historical model consistent with data presented here must explain the extreme conservation of stereospecificity in malate and lactate dehydrogenases and the divergence of stereospecificities in alcohol dehydrogenase and HMG-CoA reductases. There are several biochemical rationales that might be the basis for an assumption that stereospecificity and/or substrate specificity is more functionally contrained from drifting in the former than in the latter pair of enzymes. For example, to account for the absence of stereochemical diversity in MDH and LDH, one might assume that the deletion of MDH and LDH is lethal, while the deletion of Adh and HMG-CoA reductase is "sub-lethal", or lethal only in certain environments. Thus, stereochemical diversity arises in the latter pair, but not the former. Similar arguments might be constructed with evolutionary time as a parameter. For example, we might assume that there was a period of time where a deletion of the Adh or the HMG-CoA reductase genes was not lethal; in parallel, we might also assume that throughout evolutionary history deletion of the MDH gene was always essential in all organisms. In the time when Adh was not essential, it drifted stereochemically or a new dehydrogenase with opposite stereospecificity arose to replace it when it became needed again. In contrast, the "essential" MDH never had the opportunity to drift stereochemically. Such revised historical models are distinctly ad hoc and are extremely difficult to square with facts. For example, it is clear that there are combinations of organisms and environments where the loss of the MDH gene is *not* lethal. At best, such historical models are unpredictive without independent measures of the "essentialness" of an enzyme. These are difficult, but not necessarily impossible, to construct.

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(23) Wu, Y.-D.; Houk, K. N. *J. Am. Chem. Soc.* **1987**, *109*, 2226–2227.

(24) Glasfeld, A.; Zbinden, P.; Dobler, M.; Benner, S. A.; Dunitz, J. D., submitted.

(25) Locally diasteromeric and locally enantiomeric in this context are a matter of degree, since all transition states with configurational differences are diastereomeric in an enzyme containing L-amino acids. Here, we refer to a model for the transition state that contains only the moieties covalently connected to the reaction centers. Of course, as NADH has several chiral centers, the transition states leading to opposite stereoselectivities of any dehydrogenase are diasteromeric. Indeed, it is the diastereotopicity of the 4′-hydrogens on NADH, "caused" by the asymmetric center at C-1 of the ribose, that is the basis of the functional model discussed here. However, in the case of ethanol dehydrogenases, which "just happen" to catalyze a reaction with an intermediary redox potential, selection of a particular stereospecificity based on this diastereomerism is presumed to be absent. Hence, for this discussion, discussions of local chirality may focus on those atoms bound directly to the reaction centers.

(26) In a model of the transition state where acetaldehyde and nicotinamide are bridged by a hydrogen, the faces of the two substrates are such as to permit the largest group of each to lie between the smallest and middle-sized groups of the other.

(27) George, J. M.; Orr, J. C.; Renwick, A. G. C.; Carter, P.; Engel, L. L. *Bioorg. Chem.* **1973**, *2*, 140.

of stereochemical imperative governing the decarboxylation of β-keto acids,[28] suggest a general hypothesis: When cryptic chirality is involved,[29] enzymes catalyzing reactions via a particular chiral transition state will not in general be selected over enzymes catalyzing the same reaction via the enantiomeric transition state; enzymes catalyzing reactions via both transition states will be found in nature. In contrast, natural selection in general will distinguish between enzymes catalyzing analogous reactions via diasteromeric transition states, and only a single diastereomeric transition state will be found in naturally occurring enzymes.

Examples of nature of enzymes operating by locally enantiomeric transition states include citrate synthase,[30] certain decarboxylases,[28] and the alcohol dehydrogenases discussed here. Diasteromeric transition states are generally found in nature only when they are needed to make chiral compounds. Thus, while only limited data support this generalization at present, this general statement is worthy of experimental investigation.

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(29) This comment concerning "cryptic" chirality has some significance. In synthesizing molecules that are chiral without isotopic substitution, enzymes must have evolved an active site that synthesizes the desired enantiomer, that is, the enantiomer that is used in the next step of the metabolic pathway. This demand for stereospecificity due to metabolic coupling, discussed at length by Hanson and Rose, imposes an obvious constraint on the structure of enzymatic transition states. While the *particular* chirality may be nonfunctional (e.g, an organism making D-lactate may not be intrinsically less fit than one making L-lactate), the chirality is expected to drift more slowly than the stereospecificity of any individual enzyme, as changing the choice requires the simultaneous evolution of several enzymes that share the chiral compound as a substrate. Analogous arguments might be made to explain the universality of L-amino acids as building blocks of proteins and D sugars as building blocks of nucleic acids.

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Comparison of Carboxypeptidase A and Thermolysin: Inhibition by Phosphonamidates

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Abstract: The binding of the intact phosphonamidate inhibitor *N*-[[[(benzyloxycarbonyl)amino]methyl]hydroxyphosphinyl]-L-phenylalanine (ZGP′; K_i = 90 nM), a possible transition-state analogue of the dipeptide substrate Cbz-Gly-L-Phe, to the active site of carboxypeptidase A (CPA) is described. X-ray crystallographic analysis of the enzyme–inhibitor complex provides a well-resolved structure at 2.0-Å resolution. Although our previous study of this compound with CPA yielded the structure of a hydrolyzed phosphonamidate, optimal adjustment of pH now allows the observation of the intact complex. Both phosphonamidate and phosphonate-derived inhibitor designs have realized success toward the inhibition of zinc proteases, and their binding stereochemistry to the active sites of CPA and the related zinc endoprotease thermolysin (TLN) is summarized and considered in light of a common hydrolytic mechanism. Interestingly, for both CPA and TLN those phosphonamidate inhibitors that have P_1 glycine residues display anomalous binding modes relative to those inhibitors that have phenylalanine side chains in this position. In the current study with CPA, the CbZ-Gly moiety of ZGP′ occupies the S_1 hydrophobic side-chain cleft instead of binding in the main active site groove. The anomalous binding mode of ZGP′ leads to questions regarding its classification as an analogue of an intermediate or transition state, at least with regard to binding in the S_1 subsite.

The zinc proteases comprise a class of enzymes, intriguing from mechanistic and biological perspectives, of which carboxypeptidase A (CPA; peptidyl-L-amino acid hydrolase, EC 3.4.17.1) is perhaps the prototypical example. First isolated and characterized in 1929 by Waldschmidt-Leitz and Purr,[1] this exopeptidase is secreted by the pancreas in mammals, and its biological function is the hydrolysis of C-terminal amino acids from polypeptide substrates. Certain aspects of the catalytic mechanism of CPA have been the object of much discussion and have been emphasized in the reviews.[2-5] Thermolysin (TLN), a zinc endoprotease, is an important zinc enzyme apparently related to CPA through convergent evolution. The high-resolution X-ray structures of native CPA[6] and TLN[7] have allowed detailed analyses and comparisons of the two zinc proteases. Similarities in the active sites of these two otherwise unrelated enzymes have been invoked to imply similarities in their mechanisms of catalysis.[3,8,9]

An understanding of the zinc protease mechanism(s) is important from more than just a scientific standpoint, since several zinc proteases of unknown structure (e.g., angiotensin-converting enzyme, collagenase, enkephalinase) serve as targets for the rational design of therapeutic agents. It is helpful, then, to rely upon the implication of at least a basic similarity among the zinc proteases of known and unknown structure: the relative geometry of catalytically important residues within their active sites is

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