Table I

<table>
<thead>
<tr>
<th>entry</th>
<th>irradiation time, min</th>
<th>sensitizer</th>
<th>% product</th>
</tr>
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<td>none</td>
<td>58.6</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>none</td>
<td>&gt;80.3</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
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<td>75.1</td>
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<tr>
<td>5</td>
<td>30</td>
<td>xanthone</td>
<td>85.8</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>xanthone</td>
<td>&gt;90.0</td>
</tr>
</tbody>
</table>

(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%) to give a black solid (53 mg, 94%). Chromatography on silica gel (dichloromethane-ethyl acetate, 9:1) provided a colorless solid (25 mg, 45%) to give a black solid (53 mg, 94%).

Table III

<table>
<thead>
<tr>
<th>entry</th>
<th>irradn time, min</th>
<th>% 24a</th>
<th>equiv of piperylene</th>
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</tr>
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<tr>
<td>6</td>
<td>60</td>
<td>25.4</td>
<td>100</td>
</tr>
</tbody>
</table>

(23 mg, 0.5%). 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 chromograph fitted with a 16-ft x 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 WL (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 chromograph.

Acknowledgment. We thank the National Institutes of Health (GM26568) and the National Science Foundation (CHE83-19474) for financial support of this work. We thank the National Science Foundation and Rensselaer Polytechnic Institute for funds to purchase the Nicolet R3m X-ray diffractometer. Address correspondence concerning X-ray diffraction analyses of 12a and 16a to R.K.K.

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

Rudolf K. Alleman, Rebecca Hung, and Steven A. Benner*

Contribution from the Laboratory for Organic Chemistry, Swiss Federal Institute of Technology, CH-8092 Zurich, Switzerland. Received December 3, 1987

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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 in general will distinguish between enzymes catalyzing analogous reactions via "locally diastereomeric" 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.

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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

2. Srivastava, D. K.; Bernhard, S. A. Biochemistry 1985, 24, 622-628
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. In a research paradigm making this distinction may 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 non-functional reasons. In such enzymes, it is most likely that sufficient time has passed for stereochemical divergence 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 non-functional 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. This organism is then examined to determine which other stereochromically 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, a stereospecificity opposite that of the alcohol dehydrogenases from mammalian species. The Drosophila Adh is sufficiently distant from other organisms to have an alcohol dehydrogenase with opposite stereospecificity suggests that other dehydrogenases from Drosophila might also have divergent stereospecificities if stereochemistry 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$^+$, [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 mmol) with [1-$^3$H]glucose-6-phosphate catalyzed by glucose-6-phosphate dehydrogenase (2 units) from *L. 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 bovine kidney (0.04 mmol) and ATP (0.5 mmol). 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 mL, 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 dihydrofate (0.2 mmol) catalyzed by dihydrofate reductase (1 unit) to give [4-$^3$H]NAD$^+$, [4(R)-$^3$H]NADH and [4(R)-$^3$H]NADPH were prepared by the reduction of [4-$^3$H]NAD$^+$ and [3H]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 0-1 M NH$_4$HCO$_3$) or by HPLC. The fractions with satisfactory ultraviolet spectra were combined and lyophilized.

**Malate Dehydrogenase.** Hexokinase (5.4 g) were suspended in buffer (K$_2$HPO$_4$ 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 x 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 (13000g, 15 min). The supernatant was concentrated with NH$_4$SO$_4$. 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 13000g (15 min) to remove a brown precipitate, and the supernatant was chromatographed on DEAE-Sephadex (0.7 x 15 cm) equilibrated in Tris buffer. The activity eluted in the first 15 mL, which was concentrated and concentrated to 1.5 mL in a Amicon ultrafiltration cell (cutoff M, 1000).

To determine the stereospecificity of malate dehydrogenase, [4(S)-$^3$H]NADH (0.1 mM) was oxidized by the malate dehydrogenase preparation (1 mL) in phosphate buffer containing oxaloacetate (2 mM). Products of the reaction were separated by thin-layer chromatography on a PEI-cellulose eluted with 0.25 M lithium chloride. The corresponding fractions were collected, extracted (1 N NH$_4$OH), 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. *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 glass bead mill (2 x 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 then centrifuged (13000g, 30 min). The supernatant was concentrated by treatment with NH$_4$SO$_4$. 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 mL and contained 4.8 units with a specific activity of 16.7 (purification 125-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 spectrophotometrically 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 $\alpha$-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 (eluants, l-butanol/acetic acid/water 4:3:1). Extraction, and counted.

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

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 stereospecificity of the isocitrate dehydrogenase.

\[
\text{NADP}^+ (0.27 \text{ mM, } 2.0 \times 10^5 \text{ counts/min}) \text{ was reduced by extract (1.2 milliliters) in imidazole buffer (90 mM, pH 8.0, 1 mL containing magnesium chloride (5 mM) and N-isocitrate (0.3 mM). This reaction was monitored spectrophotometrically.}\]

When it was complete, the pro-S hydrogen was removed from NADPH 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 H\text{H}_2\text{SO}_4.

W-X\text{2} resin. NADP+ was eluted with water, the glutamate was eluted in the presence of glutamate dehydrogenase (bovine). The reaction mixture was divided into two portions. Glucose 6-phosphate dehydrogenase activity precipitated between 40% and 80% saturation. The pelleted was redissolved in extract buffer (1.0 mL) and stored at -70 °C.

Glucose 6-Phosphate Dehydrogenase. A clarified extract from 1.2 g of flies, prepared as described, was treated with 2.0% protamine sulfate solution as above and fractionated with NH\text{H}_2\text{SO}_4. The glucose 6-phosphate dehydrogenase activity precipitated between 40% and 80% saturation. The pellet was redissolved in extract buffer (1.0 mL) and stored at -70 °C.

One extract (40 mL) was incubated with \[4\-\text{H}]\text{NADPH} (1.1 mM, 1-[\text{H}]-glucose (0.28 mM, 10 \mu\text{Ci})], hexokinase (2 units), and ATP (0.8 mM) in triethanolamine buffer (1 mL, 0.1 M, pH 7.6) containing MgCl\text{2} (5 mM). In a parallel reaction, extract (40 mL) was incubated with \[4\-\text{H}]\text{NADP}^+ (0.3 mM, 2.0 \times 10^5 \text{ counts/min}) and glucose 6-phosphate (0.33 mM) in 1 mL of triethanolamine buffer containing MgCl\text{2}. The reactions were monitored spectrophotometrically (340 nm). After further reaction ceased, NADPH was isolated by ultrafiltration, separated, and counted.

Glycerol 3-Phosphate Dehydrogenase.\textsuperscript{10} The clarified extract from 1.5 g of flies homogenized in Na\text{2}HP\text{0}_4 buffer (10 mM, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mM phenylmethylsulfonfyl fluoride) was incubated for 25 min at 50 °C and centrifuged (20000g, 20 min) to remove denatured 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\-\text{H}]\text{NADH} (0.28 mM, 4.0 \times 10^3 \text{ cpm}) \text{ was incubated with } 200 \text{ gtl of extract at } 50 °C \text{ and centrifuged (20000g, 20 min) to remove denatured protein. The extract was stored at 20 °C.}

Alcohol Dehydrogenase. Alcohol dehydrogenase from Drosophila was prepared as previously described\textsuperscript{2} and used to catalyze the reduction of acetdehyde with \[4\-\text{H}]\text{NADH} to yield 1-tritiated ethanol, which was characterized as 1-tritiated ethanol. The labeled ethanol was recovered and reoxidized by NAD\text{2} in the presence of semicarbazide with alcohol dehydrogenase from yeast, known to transfer the pro-S hydrogen of ethanol. After the reaction was complete, the acetdehyde semicarbazone was diluted with carrier, identified, isolated by thin-layer chromatography, and counted. The product NAD\text{2} 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.

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.

<table>
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<td>pro-R</td>
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<td>pro-S</td>
<td>pro-S</td>
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</table>

Discussion

Two types of models may explain the stereospecificity of dehydrogenases dependent on nicotinamide cofactors.\textsuperscript{3} 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 selectale function for stereo-specificity. 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.\textsuperscript{7}

It is clear that stereospecificity in dehydrogenases is not drifting, at least not very rapidly. For example, lactate dehydrogenases from 15 organisms, including 4 l-lactate dehydrogenases and 11 l-tartrate dehydrogenases, all transfer the pro-R hydrogen from NADH.\textsuperscript{11} Likewise, male dehydrogenases from plants, animals, archaebacteria, Drosophila (reported here), and eubacteria all transfer the pro-R hydrogen.\textsuperscript{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 male 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 all organisms that act on the same substrate from different organisms displaying the same catalytic activity have the same stereospecificity ("Bentley's first rule").\textsuperscript{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;\textsuperscript{11} in Drosophila, this enzyme transfers the pro-S hydrogen.\textsuperscript{12}


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 postulating the existence of two ancestral alcohol dehydrogenases, one selecting the \( \text{pro-R} \) hydrogen, the other the \( \text{pro-S} \) 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 power 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 five malate dehydrogenases from horse, yeast, and \( Drosophila \) 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 instance, it could be argued that the catalytic domain of these dehydrogenases is the sole determinant of stereospecificity. Thus, while the dinucleotide binding domains of \( \text{Adh} \) 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 \( \text{Adh} \) 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 uncontrovertible; 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,\(^{19}\) even this prediction must be so ambiguous as to be uncontrovertible.

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 \( \text{pro-R} \) hydrogen, while enzymes optimally evolved to reduce stable carbonyls have evolved to transfer the \( \text{pro-S} \) hydrogen. Neither stereospecificity is strongly preferred in enzymes acting on carbonyl substrates with intermediary redox potential; \( K_{\text{eq}} \) refers to an equilibrium constant (\( [\text{NADH}] \)/[carbonyl][\( \text{H}^{+} \])/[\( \text{NAD}^{+} \)][alcohol]) of \( \sim 10^{-11} \) M. The divergent stereospecificities of ethanol dehydrogenases, with substrates with \( K_{\text{eq}} = 8 \times 10^{-12} \) 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.\(^{2}\) 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.\(^{4}\) 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 \( K_{\text{eq}} \) in the \( \text{"pro-R"} \) 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...
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postulate another pair of ancestral dehydrogenases, this time with the same stereospecificities purely by chance. The functional model is concerned with the peculiarities expected of functional models. If they are not homologous, the common stereospecificity conveyed 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 being non-convergent possibilities 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, 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, 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. Their evolutionary interrelatedness is unknown. However, either they are related or they are not. If they are not, 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 dehydrogenases.

Of course, these arguments do not prove the functional model based on the redox potential of the natural substrate; and this model could certainly be contradicted by experimental data yet to emerge. However, we have transformed model results into an experimental stimulus for experimental test. This statement seems especially true in light of recent experimental work showing analogous stereochemical behavior in model systems, computational work on the chemical


(25) L-locally diasteromeric and locally enantiomeric in this context are a matter of degree, since all transition states with configurational differences are diasteromeric in an enzyme containing L-amino acids. Here, we refer to a model for the transition state that contains only the moieties covalently bound to a model for the transition state that contains only the moieties covalently bound to the natural substrate is such that no strong imperative exists for the drift of substrate and stereospecificity is not the rule in dehydrogenases. 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 constrained 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 in 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 when ethanol dehydrogenases on NADH or the HMG-CoA reductase gene was lethal; in parallel, we might assume that both homologous and non-homologous, which were deleted to the best of our knowledge 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 would result in a dead organism. At best, such historical models are unprecise without independent measures of the "essentialness" of an enzyme. These are difficult, but not necessarily impossible, to construct.

of stereochemical imperative governing the decarboxylation of β-keto acids,26 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 diastereomeric transition states, and only a single diastereomeric transition state will be found in naturally occurring enzymes.

Examples of nature of enzymes operating by locally enantio-meric transition states include citrate synthase,30 certain de-carboxylases,28 and the alcohol dehydrogenases discussed here. Diastereomeric 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.

Acknowledgment. We are indebted to the Swiss National Science Foundation, U.S. National Science Foundation, NIH (GM 37702), Searle Foundation, ETH, Sandoz, Ciba-Geigy, and Hoffmann-La Roche for financial support.

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]hydroxy-phosphinyl]-l-phenylalanine (ZGP; K = 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 endopeptidase thermolysin (TLN) is summarized and considered in light of a common hydrolytic mechanism. Interestingly, for both CPA and TLN those phosphonamidate inhibitors that have P1 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 S1 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 S1 cleft.

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 endopeptidase, is an important zinc enzyme apparently related to CPA through convergent evolution. The high-resolution X-ray structures of native CPA6 and TLN7 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.3,4,9

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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(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.