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The stereospecificity of the ferrous-ion-dependent alcohol dehydrogenase from *Zymomonas mobilis*

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Alcohol dehydrogenase from *Zymomonas mobilis* has been found to transfer the *pro-R* hydrogen of NADH to acetaldehyde. This is the first report of the stereospecificity of a dehydrogenase in the mechanistic and structural class of Fe^{2+}-dependent alcohol dehydrogenases and offers an opportunity to expand mechanistic hypotheses relating stereospecificity, reaction mechanism and reaction thermodynamics in dehydrogenases.

The stereospecificity of dehydrogenases dependent on nicotinamide cofactors is a controversial paradigm illustrating how experiment and logic can help distinguish functional and non-functional behavior in enzymes [1 – 3]. Central to this controversy is a functional model for the stereospecificity of dehydrogenases that catalyze the reduction of simple carbonyl compounds [1, 2]. The model proposes that the choice of hydrogen is determined by natural selection and correlates with the redox potential of the substrate the enzyme has evolved to transform [4]. Thus, enzymes that have evolved to reduce thermodynamically unstable carbonyls generally transfer to *pro-R* (or, in the early literature, the A) hydrogen of NADH, while enzymes evolved to reduce thermodynamically stable carbonyls transfer to *pro-S* (or B) hydrogen of NADH (Table 1).

One of the principal limitations of this functional model is that it applies only to redox reactions which interconvert alcohols and carbonyl compounds. This paper focuses on experimental and logical efforts to extend the model to classes of enzymes that lie outside its original scope [1, 4]. We consider the presence and role of zinc and ferrous metal ions at the active site of certain dehydrogenases and their relevance to predictions of stereospecificity, based on functional and historical considerations, the latter most recently discussed by Schneider-Bernlöhr and her colleagues [3].

According to the functional model, the redox potential of the natural substrate is the main determinant of stereospecificity in a dehydrogenase. For example, enzymes evolved to interconvert ethanol and acetaldehyde act on a substrate of intermediary stability. The equilibrium constant for the overall reaction lies at the break point between *pro-R* and *pro-S* specificities (Table 1). Thus, the functional model predicts that stereochemical diversity might be found among enzymes oxidizing ethanol.

Such diversity was recently discovered. Alcohol dehydrogenase from yeast is *pro-R* specific, but the enzyme from *Drosophila* is *pro-S* specific [2] (this reference provides detailed evidence that ethanol is the natural substrate for *Drosophila*, alcohol dehydrogenase). Such stereochemical diversity disobeys a generalization known as Bentley's first rule, which states that dehydrogenases from different organisms catalyzing the same reaction do so with the same stereospecificity [5]. For example, malate dehydrogenase from organisms as diverse as archaebacteria eubacteria, plants and animals all transfer the *pro-R* hydrogen.

However, these two alcohol dehydrogenases differ also in their mechanism. The enzyme from yeast has long been known to require a zinc ion for catalysis [6]. The enzyme from *Drosophila* lacks a zinc atom. These facts recently prompted Schneider-Bernlöhr and her colleagues to extend a historical model, originally developed by Jornvall and his coworkers [6], to account for the different stereospecificities of *Drosophila* and yeast alcohol dehydrogenases and their close homologs [3]. The model divides alcohol dehydrogenases into two divergent pedigrees [6]: a 'high-molecular-weight' type (35 – 40 kDa) which contains zinc in the active site and is *pro-R* specific, and a 'low-molecular-weight' type (25 kDa) lacking metal ions which is *pro-S* specific.

The model then proposes that the predominant factor in explaining dehydrogenase specificity is membership in one of these two divergently evolved subclasses. To the extent that the model makes predictions, the model might predict that long-chain zinc-dependent enzymes are homologous, as are short-chain-polyol dehydrogenases, so that any newly isolated activity belonging to one of the two size and mechanism groups will share ancestry and stereochemistry.

Relevant to this discussion are the isozymes of alcohol dehydrogenase found in *Zymomonas mobilis*, ZADH I and ZADH II. The former is as a zinc-dependent enzyme [7] with strong similarity to other bacterial alcohol dehydrogenases [8, 9] and therefore to the zinc-dependent enzymes of yeast, mammals and plants [10]. In contrast, ZADH II is a novel type of alcohol dehydrogenase. It has a high specificity for ethanol [7], requires ferrous ion for activity [8], and shows no obvious sequence similarity to other dehydrogenases, except ADH IV from yeast, which also appears to require a ferrous ion for catalytic activity [11, 12].

Alcohol dehydrogenases dependent on ferrous ion might best be viewed as members of a third, independent, pedigree of enzymes that convergently evolved to act on ethanol. The

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Abbreviations. ADH, alcohol dehydrogenase; ZADH, alcohol dehydrogenase from *Zymomonas mobilis*.

Enzyme. Alcohol dehydrogenase (EC 1.1.1.1).

Table 1. *List of alcohol dehydrogenases sorted by equilibrium constant to demonstrate the correlation of redox potential with stereochemistry* Note that stereochemical diversity exists near the discontinuity between A- and B-specific enzymes. A stereospecificity corresponds with *pro-R* specificity; and B with *pro-S*. For references, see [1]; pK_{eq}, p*K* at equilibrium

Enzyme	EC no.	pK_{eq}	Specificity
Glyoxylate reductase	1.1.1.26	17.5	A
Glyoxylate reductase ($NADP^+$)	1.1.1.79	17.5	A
Tartronate semialdehyde reductase	1.1.1.60	13.3	A
Glycerate dehydrogenase	1.1.1.29	13.3	A
Glycerol dehydrogenase	1.1.1.72	12.8	A
Hydroxypyruvate reductase ($NADP^+$)	1.1.1.81	12.4	A
Malate dehydrogenase	1.1.1.37	12.1	A
Malate dehydrogenase ($NADP^+$)	1.1.1.82	12.1	A
'Malic' enzyme	1.1.1.38	12.1	A
'Malic' enzyme ($NADP^+$)	1.1.1.40	12.1	A
L-Lactate dehydrogenase	1.1.1.27	11.6	A
D-Lactate dehydrogenase	1.1.1.28	11.6	A
Alcohol dehydrogenase (yeast)	1.1.1.1	11.4	A
Alcohol dehydrogenase (*Drosophila*)	1.1.1.1	11.4	B
Glycerol dehydrogenase	1.1.1.6	11.3	A
Glycerol-3-phosphate dehydrogenase	1.1.1.8	11.1	B
Homoserine dehydrogenase	1.1.1.3	10.9	B
Carnitine dehydrogenase	1.1.1.108	10.9	B
3-Hydroxyacyl-CoA dehydrogenase	1.1.1.35	10.5	B
Cinnamyl-alcohol dehydrogenase	1.1.1.195	9.1	A
3-Hydroxybutyrate dehydrogenase	1.1.1.30	8.9	B
3β-Hydroxysteroid dehydrogenase	1.1.1.51	8.0	B
Estradiol 17β-dehydrogenase	1.1.1.62	7.7	B
Testosterone 17β-dehydrogenase	1.1.1.64	7.6	B
3-Oxoacyl-[acyl-carrier-protein] dehydrogenase	1.1.1.100	7.6	B
17β-Hydroxysteroid dehydrogenase	1.1.1.51	7.6	B

historical model makes no predictions regarding stereospecificity in such an independent lineage. Nevertheless, a third lineage complicates historical models, as a postulate of multiple ancestors in one class of dehydrogenases makes the assumption that enzymes in other classes are descendents from a single ancestral protein more tenuous, and that postulate is generally necessary to explain Bentley's first rule.

Functional models may also be complicated by a third mechanistic class of enzymes. As stereochemical imperatives are ultimately based on the underlying enzymatic reaction mechanism, empirical correlations valid for one mechanistic class need not hold for a different class. Nevertheless, one may try to extend the mechanistic principles themselves; the new class then becomes an independent test of these underlying hypotheses. Here, a functional hypothesis might be that enzymes that employ a metal ion in the active site are functionally best adapted when they transfer the *pro-R* hydrogen.

The correlation identified by Schneider-Bernlöhr relating metal ion dependency and *pro-R* specificity is consistent with this view [3]. Further, ZADH II permits an independent test of such a hypothesis. The enzyme is not obviously homologous to the zinc-dependent enzymes [11], yet it contains a metal ion which appears to fill the same mechanistic role as the zinc in zinc-dependent dehydrogenases [8]. If function determines stereospecificity in metal-dependent dehydrogenases, iron-dependent dehydrogenases should have the same stereospecificity as zinc-dependent enzymes.

We report here that alcohol dehydrogenase II from *Zymomonas* catalyzes the transfer of the *pro-R* hydrogen of NADH. This is the first report of the stereospecificity of an iron-dependent alcohol dehydrogenase. Further, we present a functional explanation for this result and the data discussed by Schneider-Bernlöhr's historical model. While the data still do not permit a final verdict regarding the relative merits of the functional and historical models discussed here, a dialectic between the two is a necessary first step to stimulate thought and experimental work in this area.

EXPERIMENTAL PROCEDURES

Zymomonas mobilis (ATCC no. 10988) was obtained from the Deutsche Sammlung von Mikroorganismen and grown according to the method of Wills [5]. Cells were harvested when the total absorbance at 600 nm reached 1.1; 2 l media yielded 7.5 g wet cells. Cells were stored for one month at $-70°C$ prior to disruption.

Zymomonas cells (2 g) suspended in 10 ml 10 mM sodium phosphate pH 7.0, containing 1 mM dithiothreitol and 1% phenylmethylsulfonyl fluoride, were lysed by gentle sonication using a Heat Systems Ultrasonics model W-370 sonicator. Cellular debris was removed by centrifugation ($40000 \times g$, 15 min, 4°C) and the crude extract was loaded onto a Pharmacia blue-Sepharose CL-6B column (2.5×15 cm) pre-equilibrated with elution buffer (10 mM sodium phosphate, pH 7.0, 1 mM dithiothreitol). ZADH II was eluted with a gradient of 0–1 mM NADH in elution buffer. Fractions containing activity were combined, concentrated by ultrafiltration under N_2 and dialyzed against 4 l 10 mM sodium phosphate, pH 7.0, containing 300 mM sodium sulfate and 1 mM dithiothreitol. ZADH II was identified by its inability to catalyze the oxidation of *n*-butanol with NAD^+ and by its migration further to the anode, under native polyacrylamide electrophoresis conditions at pH 8.8, relative to yeast ADH I. The alcohol dehydrogenase appeared as a single band on activity staining.

Tritium-labelled cofactors were prepared as described previously [13]. Acetaldehyde was reduced by (*S*)-[4-^{3}H]NADH and (*R*)-[4-^{3}H]NADH in the presence of ZADH II in 50 mM phosphate buffer, pH 7.0. Volatile material was separated from cofactors and salts by bulb-to-bulb distillation. The radioactivity in the two fractions was measured and the relative activities determined.

RESULTS AND DISCUSSION

ADH II from *Zymomonas mobilis* catalyzes the transfer of the *pro-R* hydrogen from NADH. Enzyme-catalyzed oxidation of (*S*)-[4-^{3}H]NADH by acetaldehyde produced ethanol containing less than 2% of the total radioactivity in the volatile fraction. The corresponding oxidation of (*R*)-[4-^{3}H]NADH by acetaldehyde yielded ethanol containing 85% of the activity in the volatile fraction.

This experiment does not by itself provide a critical test of the functional model discussed above. Because the equilibrium constant for ethanol oxidation lies near the discontinuity between *pro-S*- and *pro-R*-type reactions in the correlation (Table 1), neither possible experimental outcome would necessarily contradict the model. Nor does the result by itself contradict historical models; however, a third independent lineage of alcohol dehydrogenases must be postulated to account for the result.

However, the *pro-R* specificity of ZADH II does have potential implications with respect to the mechanistic hypotheses underlying the functional model's explanation of the correlation. In zinc-dependent alcohol dehydrogenases, the carbonyl substrate has long been presumed to coordinate to the metal ion prior to reduction [14]. This coordination presumably destabilizes the carbonyl substrate by enhancing the electrophilicity of the carbonyl carbon. Similarly, the metal ion stabilizes the alkoxide anion that is the putative first product in the reduction reaction.

To the extent that the metal ion is a more effective electrophile than the electrophile used in non-metal dehydrogenases (most probably the proton of a general acid), the internal equilibrium constant of the acetaldehyde/ethanol couple (that between the two ternary complexes enzyme—acetaldehyde—NADH and enzyme—ethanol—NAD^+) [15] is expected to be shifted more in favor of the products when compared to the internal equilibrium constant for the acetaldehyde/ethanol couple in non-zinc enzymes.

The functional model argues that the conformation of NADH transferring the *pro-R* hydrogen is a weaker reducing agent than the conformation transferring the *pro-S* hydrogen. Further, the model argues that natural selection choses the conformation of the cofactor whose redox potential more nearly matches the redox potential of the bound substrate. Thus, the mechanistic hypotheses underlying the functional model argue that the correlation in metal-dependent dehydrogenases between stereospecificity and external redox potential of substrate will be different from the correlation for dehydrogenases not dependent on metal ions. Specifically, the break point in the correlation will be shifted towards higher equilibrium constants; in cases where both metal and non-metal dehydrogenases are known to act on the same substrate, some examples are expected where the non-metal enzyme transfers the *pro-S* hydrogen, while the metal-dependent dehydrogenase transfers the *pro-R* hydrogen.

The *pro-R* specificity is consistent with this hypothesis if, as proposed by Neale et al. [8], the ferrous ion serves a role analogous to that of the zinc ion in horse liver alcohol dehydrogenase. If this proposal is correct, here as in yeast alcohol dehydrogenase, the metal is expected to destabilize bound carbonyl groups relative to bound alcohols, leading to a preference for the *pro-R* hydrogen.

In fact, all enzymes believed to contain a catalytic zinc catalyze the transfer of the *pro-R* hydrogen of NADH. Care must be taken in applying this model to other types of metal-dependent dehydrogenases, such as those that catalyze several successive reactions. For example, L-'malic' enzyme catalyzes the oxidative decarboxylation of L-malate to produce pyruvate. Mg(II) or Mn(II) participates in this reaction. However, the metal ion probably participates in the decarboxylation step, not in the oxidation step. L-'Malic' enzyme transfers a hydride equivalent to the *re* face of NAD^+, a fact that naively appears to confirm the model proposed above. However, if the metal is not involved in the oxidation step, the confirmation is deceptive. Similar facts (and arguments) apply to isocitrate dehydrogenase. Here, as elsewhere, it is imprudent to examine enzymes catalyzing multistep reactions as a test for this hypothesis without a detailed understanding of the mechanism. In several cases, these enzymes act on substrates of intermediary stability with redox potentials near the break point in the correlation (Table 1). However, in one case (cinnamyl-alcohol dehydrogenase), the metal-dependent dehydrogenase transfers the *pro-R* hydrogen even though the correlation (Table 1) would predict that the *pro-S* hydrogen should be transferred.

Initially, the correlation was proposed to exclude enzymes acting on α,β-unsaturated carbonyl compounds; the rationale for this exclusion was that such substrates might be reduced by different mechanisms (perhaps involving a radical intermediate) [1, 2]. However, the unexpected stereospecificity of cinnamyl alcohol dehydrogenase is also consistent with the participation of a zinc ion in the active site [16], which is expected to promote selection for *pro-R* specificity on the grounds given above. In this view, the presence or absence of a catalytic metal ion could be stereochemically significant for functional rather than historical reasons.

The functional hypothesis proposed here contrasts directly with the historical model proposed by Schneider-Bernlöhr and her colleagues [3]. Both hypotheses are based only on a small number of examples and direct participation of a metal in the active site of several others remains to be proven. Further, structural features other than the presence of a metal ion could influence the correlation between equilibrium constant and stereospecificity, and we do not yet understand all the effects that could contribute to the apparent preference of enzymes for the *pro-R* hydride for reducing unstable carbonyls. Thus, the model is a starting point, rather than a final conclusion, from which we can begin to understand the link between stereochemistry and function in enzymology and the broader questions relating enzymatic behavior and natural selection.

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