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Aldose Reductase: Model for a New Paradigm of Enzymic Perfection in Detoxification Catalysts†

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Aldose reductase (EC 1.1.1.21; ALR2[1]) is a broad specificity NADPH-dependent aldehyde reductase that appears by the accepted criteria for "enzymic perfection" to be a rather poor enzyme indeed. Yet, the wide distribution of ALR2 and related enzymes among mammalian tissues (Markus et al., 1983; Wirth & Wermuth, 1985; Mathur & Grimshaw, 1986), as well as in birds, amphibians, plants, fungi, and bacteria (Davidson et al., 1978; Carper et al., 1987; Bohren et al., 1989; Schade et al., 1990; Bartels et al., 1991), suggests that these are highly evolved enzymes with a sound mechanistic and physiologic basis for their apparently inefficient kinetic properties. The idea of perfection introduced by Knowles and Albery (Albery & Knowles, 1976, 1977; Knowles & Albery, 1977) and subsequently extended (Chin, 1983; Burbaum et al., 1989; Burbaum & Knowles, 1989; Pettersson, 1989) holds that a highly evolved enzyme will have a turnover rate limited by the rate of diffusion of substrates to and products away from the active site and further that the internal equilibria for bound states and transition states will be balanced so that no step contributes an inordinate amount to the overall reaction progress. A parallel view holds that the intrinsic binding energy of substrates will be optimally utilized to stabilize the transition state for the reaction relative to the ground state, so that any excess intrinsic binding energy used to enhance binding beyond the point where K_m becomes equal to the physiological substrate concentration is considered to be wasted (Fersht, 1974; Jencks, 1975). ALR2 apparently violates both tenets by expending much of the available intrinsic binding energy in the very tight binding of both $NADP^+$ (0.08 μM) and NADPH (0.05 μM) (Grimshaw et al., 1990b). As a direct result, V/E_t for reaction in *both* directions is apparently limited by the rate of nucleotide release or rather the E·nucleotide isomerization step that precedes nucleotide release (Grimshaw et al., 1990a; Kubiseski et al., 1992). Yet, ALR2 can instead be viewed as the model for a new paradigm of enzymic perfection for catalysts involved in detoxification of a broad range of substrates containing a particular functional group. Examples drawn from the lactate dehydrogenase and alcohol dehydrogenase families will be discussed in terms of the ALR2 model, and suggestions will be made for extension of this paradigm to a variety of detoxification and salvage enzymes.

Comparison of Aldose Reductase with A_4 Lactate Dehydrogenase. In order to provide a frame of reference for understanding the peculiar kinetic properties of ALR2, I have chosen as a prototype for comparison the monospecific NADH-dependent reductase, dogfish muscle lactate dehydrogenase (A_4-LDH). The reduction of pyruvate to L-lactate mediated by A_4-LDH and the ALR2-catalyzed interconversion of glycolaldehyde and ethylene glycol both follow an ordered bi-bi kinetic mechanism, as shown in Scheme I, where E is enzyme, NH and N are NAD(P)H and $NAD(P)^+$, and RO and ROH are aldehyde/ketone and alcohol, respectively. For simplicity, isomerization steps involving intermediate enzyme complexes are not shown, although such steps are known to be important in the overall kinetic mechanisms of both ALR2 (Grimshaw et al., 1990b; Kubiseski et al., 1992) and A_4-LDH (Holbrook et al., 1975; Waldman et al., 1986). The key parameters for the reactions mediated by these two enzymes are listed in Table I and are presented as a free energy diagram in Figure 1, where I have assumed for convenience a 1.0 M standard state for each substrate and product and a standard free energy for E + NH + RO equal to zero.

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[1] Abbreviations: ALR2, aldose reductase; ADH, alcohol dehydrogenase; *Hs*ADHβ, human (*Homo sapiens*) liver ADH, $\beta_1\beta_1$ homodimer; *Sc*ADH1 and *Sc*ADH2, ADH isozymes I and II from *Saccharomyces cerevisiae*; LDH, lactate dehydrogenase; A_4-LDH, dogfish muscle or rabbit muscle LDH, A_4 homotetramer; C_4-LDH, mouse testes LDH, C_4 homotetramer.

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Scheme I

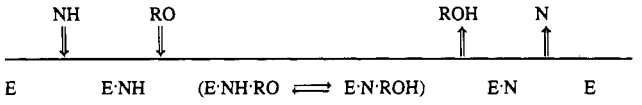


Table I: Comparison of Kinetic Parameters for Aldose Reductase and Lactate Dehydrogenase Isozymes

parameter	ALR2[a]	A_4-LDH[b]	C_4-LDH[c]
V_{RO}/E_t (s^{-1})	0.18	230	12
$V/K_{RO}E_t$ (M^{-1} s^{-1})[e]	4.5×10^3 (2.2×10^5)[d]	3.4×10^5	4.0×10^5
V_{ROH}/E_t (s^{-1})	0.02	50	3.6
$V/K_{ROH}E_t$ (M^{-1} s^{-1})	5×10^{-3}	833	730
$K_{i\ NAD(P)}$ (μM)	0.08	350	10
$K_{i\ NAD(P)H}$ (μM)	0.05	8	0.7
ratio: $K_{i\ NAD(P)}/K_{i\ NAD(P)H}$	1.6	44	14
ratio: $(V/K_{RO})/(V/K_{ROH})$	875000	410	550
Haldane[f]	1.4×10^6	1.8×10^4	7.8×10^3
V/K ratio: log fraction of K_{eq}' (%)	97	61	70

[a] Bovine kidney aldose reductase with glycoaldehyde/ethylene glycol and NADPH/$NADP^+$ as substrates, 50 mM K-Mops buffer (pH 8.0), 15 °C, adapted from Grimshaw et al. (1990b). [b] Dogfish muscle A_4-LDH with pyruvate/L-lactate and NADH/NAD^+ as substrates, 100 mM Na-P_i buffer (pH 7.5), 15 °C, adapted from Burgner et al. (1978). [c] Mouse testes C_4-LDH with pyruvate/L-lactate and NADH/NAD^+ as substrates, 100 mM Na-P_i buffer (pH 7.5), 25 °C, values estimated from the results of Goldberg (1972), Blanco et al. (1976), and Lee et al. (1977). [d] Value for D-glyceraldehyde. [e] Based on free carbonyl species after correction for hydration. [f] Haldane: $K_{eq}' = V_{RO}K_{ROH}K_{i\ NAD(P)}/V_{ROH}K_{RO}K_{i\ NAD(P)H}$, where the apparent equilibrium constant ($K_{eq}' = K_{eq}[H^+]$) includes the hydronium ion concentration. Values shown are calculated from the experimental parameters; reported K_{eq} values (corrected for hydration) are 1.2×10^{14} M^{-1} for 2-hydroxyaldehyde reduction (Kormann et al., 1972; Robinson, 1966) and 3.8×10^{11} M^{-1} for pyruvate reduction (Hakala et al., 1956).

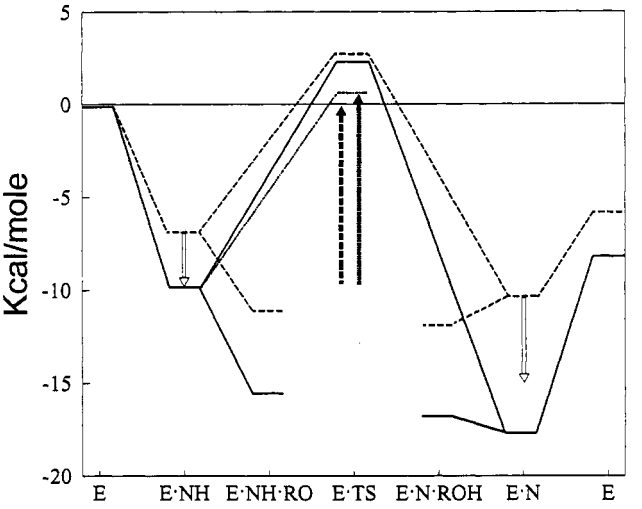


FIGURE 1: Free energy diagram comparing bovine kidney ALR2 (glycolaldehyde, solid line; D-glyceraldehyde, short dashed line) and dogfish muscle A_4-LDH (pyruvate, long dashed line) catalysis of the normal forward and reverse reactions, where E is enzyme, NH and N are NAD(P)H and $NAD(P)^+$, RO and ROH are aldehyde/ketone and alcohol, and TS is the transition state, respectively. Open arrows show the free energy difference ($\Delta G'_{ALR2} - \Delta G'_{LDH}$) between the binary E·N and E·NH complexes of ALR2 and A_4-LDH. Filled arrows compare $\Delta G^{\ddagger}$ for ALR2-mediated reduction of D-glyceraldehyde (short dashes) with A_4-LDH-mediated reduction of pyruvate (long dashes). See text and Table I for individual rate and equilibrium constants.

The most striking difference between the two enzymes occurs in the free energies of the binary E·nucleotide complexes. As shown (open arrows), the binary E·NH and E·N complexes of ALR2 are stabilized by an additional 2.9 and 7.3 kcal/mol, respectively, relative to the corresponding complexes of A_4-LDH, reflecting $K_{i\ NAD(P)H}$ and $K_{i\ NAD(P)^+}$ values for ALR2 that are 160-fold and 4400-fold lower than the corresponding values for NADH and NAD^+ with A_4-LDH. As noted above, this extremely tight binding is directly responsible for the 1280-fold and 2500-fold lower V/E_t values seen for ALR2 catalysis. By contrast, the rate-limiting step for pyruvate reduction has been shown to be an isomerization of the ternary E·NADH·pyruvate complex preceding hydride transfer (Holbrook et al., 1975). Yet, the free energy barrier corresponding to $V/K_{RO}E_t$ (E·NH → E·TS) is similar for the two enzymes (filled arrows) if one uses for comparison the value for ALR2-catalyzed reduction of D-glyceraldehyde which is more representative of the values observed for preferred D-aldose substrates (see below).

By binding *both* nucleotides with almost equal affinity, ALR2 is able to achieve essentially "one-way" catalysis in the direction of aldehyde reduction. Because the K_{ROH} value for ethylene glycol oxidation is so large (4 M), the ratio of V/K values for aldehyde reduction *versus* alcohol oxidation for ALR2 [i.e., $(V/K_{RO})/(V/K_{ROH}) = 875\,000$] constitutes a 97% log fraction[2] of the equilibrium constant for the overall reaction! In other words, because the dissociation constants for NADPH and $NADP^+$ are nearly equal, the Haldane expression for an ordered bi-bi kinetic mechanism ($K_{eq}' = V_{RO}K_{ROH}K_{i\ NADP^+}/V_{ROH}K_{RO}K_{i\ NADPH}$) requires that the ratio of V/K values be nearly equal to K_{eq}'. The extremely low $K_{i\ NADPH}$ value for ALR2, combined with the fact that in vivo the intracellular redox ratio favors NADPH [[NADPH]/[$NADP^+$] = 50–500 (Veech, 1987)], further ensures that ALR2 will always be saturated with NADPH. A_4-LDH, on the other hand, displays more of a balance with the V/K ratio comprising only a 61% log fraction of K_{eq}'. Consequently, given the physiological redox ratio for NAD^+ [[NAD^+]/[NADH] = 500–1000 (Veech, 1987)], the K_i ratio for A_4-LDH ($K_{i\ NAD^+}/K_{i\ NADH} = 44$) allows the enzyme to function effectively in either direction.[3]

The efficacy of ALR2 is apparent from a comparison of the catalytic efficiencies for reduction of a series of carbonyl-containing substrates of increasing chain length (Figure 2). Thus, ALR2 maintains a fairly constant $V/K_{RO}E_t$ value for reduction of aldoses containing 3, 5, or 6 carbon atoms and for *p*-nitrobenzaldehyde. On the other hand, A_4-LDH shows a rapid decline in $V/K_{RO}E_t$ value with increasing chain length, decreasing nearly 3 log units by the time one reaches 2-oxohexanoate and phenyl pyruvate. The ALR2 active site is not simply permissive, however, because 2-L-hydroxyaldoses display $V/K_{RO}E_t$ values that are consistently 15–30-fold lower than those for the natural 2-D isomers.[4] Due to the slow E·$NADP^+$ isomerization step (Grimshaw et al., 1990b; Kubiseski et al., 1992), V_{RO}/E_t for ALR2 is, within experimental error, constant across the spectrum of aldose substrates while V_{RO}/E_t for A_4-LDH decreases nearly 1.5 log units going from pyruvate to 2-oxohexanoate and phenyl pyruvate. Thus,

[2] The Haldane expression for these dehydrogenases ($V_{RO}K_{ROH}K_{i\ NAD(P)}/V_{ROH}K_{RO}K_{i\ NAD(P)H}$) can be factored into a V/K ratio [$(V/K_{RO})/(V/K_{ROH})$] and a K_i ratio ($K_{i\ NAD(P)}/K_{i\ NAD(P)H}$). The log fraction (percent) contribution of the V/K ratio to the Haldane expression is thus equal to 100(log (V/K ratio)/[log (V/K ratio) + log (K_i ratio)]).

[3] Evolution has produced two types of monospecific LDH subunits. The A_4 isozyme, which is fine-tuned for pyruvate reduction during anaerobic glycolysis in skeletal muscle, displays higher K_m values for pyruvate and L-lactate and is less susceptible to substrate inhibition by pyruvate than is the B_4 isozyme, which functions primarily to oxidize L-lactate in cardiac muscle (Kaplan et al., 1968).

[4] Because glycolaldehyde does not have an additional hydroxymethyl group attached at C2, but rather has two enantiotopic hydrogen atoms, ALR2 apparently handles this substrate as an L-2-hydroxy isomer.

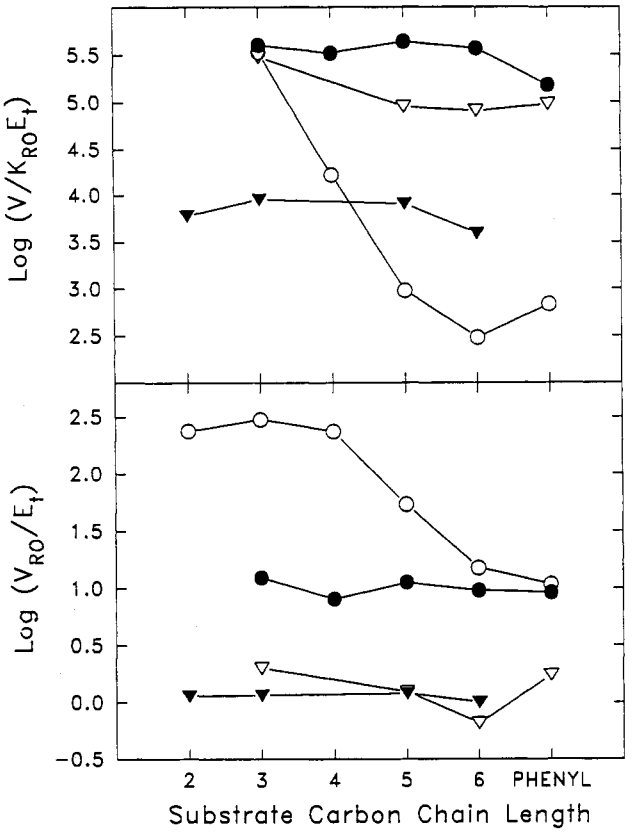


FIGURE 2: Variation of kinetic parameters for aldehyde/ketone reduction with substrate chain length. log (V_{RO}/E_t) and log ($V/K_{RO}E_t$) are plotted versus substrate chain length for bovine kidney ALR2 [2-D-hydroxyaldoses (▽), 2-L-hydroxyaldoses (▼) including glycolaldehyde] (Grimshaw et al., 1989; Grimshaw, 1991) and for 2-oxoacid reduction by rabbit muscle A_4-LDH (○) (Meister, 1950) and mouse C_4-LDH (●) (Blanco et al., 1976).

despite (or perhaps because of—see below) the extremely tight binding of both NADPH and $NADP^+$, ALR2 is a highly efficient (in $V/K_{RO}E_t$ terms), virtually unidirectional (V/K ratio) catalyst for reduction of a broad range of aldehyde substrates.

A Broad Specificity Lactate Dehydrogenase Isozyme. A similar pattern of broad substrate specificity, high catalytic efficiency in $V/K_{RO}E_t$ terms, and low turnover number is displayed by the C_4-isozyme of lactate dehydrogenase found only in testes (Blanco et al., 1976; Lee et al., 1977). As shown in Figure 2, $V/K_{RO}E_t$ for catalysis by C_4-LDH remains constant across a broad range of 2-oxoacid substrates at a value comparable to that for pyruvate reduction by the monospecific A_4-isozyme. V_{RO}/E_t for C_4-LDH-catalyzed reduction of pyruvate through 2-oxohexanoate and phenyl pyruvate is constant, albeit at a rate 30-fold below that for A_4-LDH-catalyzed reduction of pyruvate. As with ALR2, broad specificity has apparently been achieved at the "expense" of a 35-fold increase in binding affinity for NADH and an 11-fold increase in binding affinity for NAD^+, with the result that the V/K ratio now constitutes a 71% log fraction of K_{eq}', intermediate between the log fractions found for ALR2 and A_4-LDH.[5]

Monospecific and Broad Specificity Alcohol Dehydrogenase Isozymes. Comparison of kinetic parameters for yeast alcohol dehydrogenase isozyme I (*Sc*ADH1) and the $\beta_1\beta_1$-isozyme of human liver alcohol dehydrogenase (*Hs*ADHβ) reveals a similar pattern in the alcohol dehydrogenase family where a relatively monospecific enzyme binds nucleotides much less tightly than does the broad specificity counterpart. *Sc*ADH1, which is the major constitutive form in yeast, is involved in fermentative reduction of acetaldehyde to ethanol as the final step of anaerobic glycolysis (Young et al., 1982). The *Hs*ADHβ isozyme, on the other hand, is one of several broad specificity alcohol dehydrogenases found in human liver whose primary physiological role is presumed to be detoxification (metabolism) of various alcohols (Bosron et al., 1983; Wagner et al., 1983; Deetz et al., 1984; Burnell et al., 1989). This particular ADH isozyme was chosen for comparison because it displays extremely tight nucleotide binding ($K_{i\ NADH}$ = 0.19 μM) and low V/E_t values (Bosron et al., 1983) similar to ALR2 and C_4-LDH.

Table II: Comparison of Kinetic Parameters for Alcohol Dehydrogenase Isozymes

parameter	*Sc*ADH1[a]	*Hs*ADHβ[b]
V_{RO}/E_t (s^{-1})	1700	4.0
$V/K_{RO}E_t$ ($M^{-1}\ s^{-1}$)	3.1×10^6	1.2×10^5
V_{ROH}/E_t (s^{-1})	340	0.15
$V/K_{ROH}E_t$ ($M^{-1}\ s^{-1}$)	2.0×10^4	6.8×10^3
$K_{i\ NAD}$ (μM)	920	90
$K_{i\ NADH}$ (μM)	31	0.19
ratio: $K_{i\ NAD}/K_{i\ NADH}$	30	470
ratio: $(V/K_{RO})/(V/K_{ROH})$	150	18
Haldane[c]	4.6×10^3	8.4×10^3
V/K ratio: log fraction of K_{eq}' (%)	59	32

[a] Ethanol/acetaldehyde and NAD^+/NADH as substrates, 83 mM K-P_i/40 mM KCl buffer (pH 7.3), 30 °C, adapted from Ganzhorn et al. (1987). [b] Ethanol/acetaldehyde and NAD^+/NADH as substrates, 100 mM Na-P_i buffer (pH 7.5), 25 °C, adapted from Bosron et al. (1983). [c] Haldane: $K_{eq}' = V_{RO}K_{ROH}K_{i\ NAD}/V_{ROH}K_{RO}K_{i\ NADH}$, where the apparent equilibrium constant ($K_{eq}' = K_{eq}[H^+]$) includes the hydronium ion concentration. Values shown are calculated from the experimental parameters; the reported K_{eq} value for acetaldehyde reduction is 10^{11} M^{-1} (Sund & Theorell, 1963).

The differences in kinetic properties for the two alcohol dehydrogenases are again reflected in their substrate specificities. As shown in Table II, $K_{i\ NAD^+}$ and $K_{i\ NADH}$ values for the broad specificity *Hs*ADHβ are 10-fold and 160-fold lower than those for *Sc*ADH1. $V/K_{ROH}E_t$ for *Hs*ADHβ-catalyzed oxidation of a series of primary alcohols ranging in size from ethanol to hexanol is constant, with a value similar to that for *Sc*ADH1-catalyzed oxidation of ethanol, while $V/K_{ROH}E_t$ for *Sc*ADH1 decreases 30-fold across the same range of substrates (Figure 3). Because of the slow release of NADH, V_{ROH}/E_t for *Hs*ADHβ is constant over the entire range of alcohols at a level more than 3 log units below that seen for ethanol oxidation by *Sc*ADH1. A recent kinetic study has shown that V/E_t for catalysis by *Hs*ADHβ in *both* directions is limited by the rate of nucleotide release (Stone et al., 1991), while the observation of significant primary hydrogen isotope effects suggests that hydride transfer is partially rate-limiting for reaction in both directions with the yeast enzyme (Dickenson & Dickinson, 1975).

In contrast to ALR2, the liver-type alcohol dehydrogenases have evolved to operate in the *opposite*, thermodynamically unfavorable direction, i.e., alcohol oxidation. In theory, an ideal "alcohol removease" should evolve to the point where

[5] Succinic semialdehyde reductase (SSAred), which is specific for succinic semialdehyde and displays K_m values for NADPH and $NADP^+$ that are 10-fold and 120-fold greater and V_{RO}/E_t and V_{ROH}/E_t values that are 20-fold and 45-fold greater than those for ALR2, respectively (Kaufman et al., 1979; Rumigny et al., 1980), is an example of a monospecific NADPH-dependent aldoketo reductase. The V/K ratio for SSAred constitutes only a 73% log fraction of K_{eq}', which is far down from the 97% log fraction seen for ALR2 and more in line with the 61% log fraction seen for A_4-LDH.

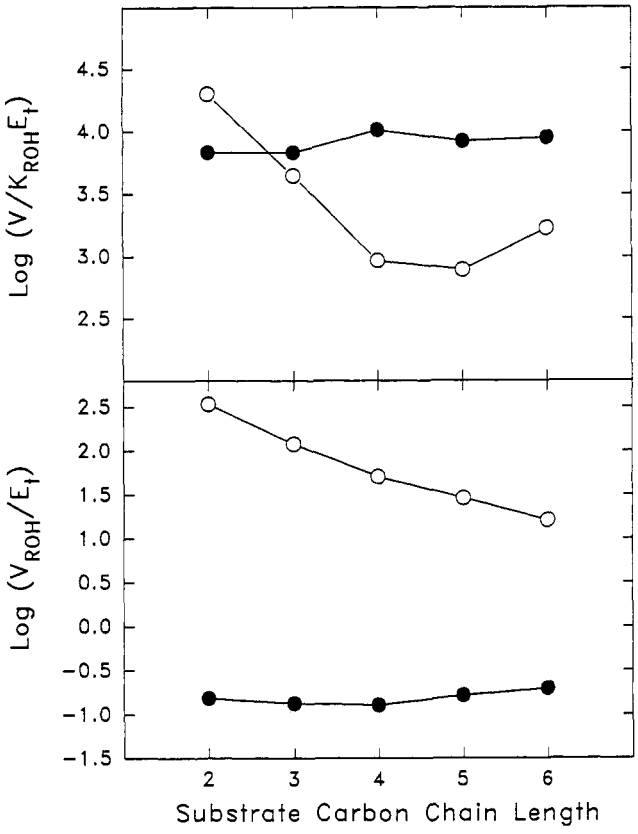


FIGURE 3: Variation of kinetic parameters for alcohol oxidation with substrate chain length. log (V_{ROH}/E_t) and log ($V/K_{ROH}E_t$) are plotted versus substrate chain length for *Hs*ADHβ (●) (Burnell et al., 1989) and *Sc*ADH1 (○) (Ganzhorn et al., 1987).

the V/K ratio optimally *offsets* the external equilibrium constant, opposite to the nearly 100% expression of K_{eq} in the V/K ratio for ALR2. By this criterion, the *Hs*ADHβ isozyme would appear to be less highly evolved than ALR2 since the V/K ratio for *Hs*ADHβ still constitutes a 32% log fraction of K_{eq}. Note, however, that the observed K_i ratio ($K_{i\ NAD^+}/K_{i\ NADPH}$ = 470) is already matched to the NAD^+ redox ratio in liver cytosol [[NAD^+]/[NADH] = 725 (Williamson et al., 1967)], which means that any further decrease in the V/K ratio would result in significant product inhibition by NADH. Thus, the *Hs*ADHβ isozyme is, in fact, ideally suited to function as a broad spectrum alcohol detoxification catalyst in liver cytosol.

Mechanistic Logic of Broad Specificity NAD(P)-Dependent Oxidoreductases. The data presented suggest that very tight binding of the nucleotide cofactor is a prerequisite for achieving the type of broad substrate specificity shown by ALR2, C_4-LDH, and *Hs*ADHβ. Moreover, the fact that a high catalytic efficiency (in V/K terms) is maintained over a broad range of substrate structures suggests that any transition-state stabilization derived from noncovalent interactions with nonreacting portions of the substrate must be quite small. Rather, these catalysts appear to be unique in their ability to derive most of their transition-state stabilization energy from interaction with the cofactor, NAD(P)H or NAD^+. Fersht has estimated that if all the intrinsic binding energy were realized, a molecule of the complexity of NADP(H) could attain a dissociation constant of 10^{-20} M (Fersht, 1985). The E·NADPH complex of ALR2 displays a K_d value of only 5 × 10^{-8} M, which still leaves 15 kcal/mol for stabilization of the transition state. However, because a great deal of intrinsic energy is actually *expressed* in the binding, these results imply that only by first binding the cofactor very tightly (in the extreme, covalently) can the enzyme maximally utilize the remaining energy.

Jencks has suggested that by binding the thermodynamically favored product(s) of the reaction less tightly than the substrate(s) an enzyme may utilize a greater proportion of the intrinsic binding energy available from the product(s) to shift the bound-state equilibrium position closer to a value of unity (Jencks, 1975). This appears to be the case for the *Hs*ADHβ, where NAD^+ is bound much less tightly than NADH and the K_m values for the alcohol substrate and aldehyde product are similar (cf. Table II). Experimental determination of the bound-state K_{eq} for the related EE-isozyme from equine liver showed a value near 0.15 (Sekhar & Plapp, 1990). For ALR2, however, NADPH and $NADP^+$ bind with nearly equal affinity, and it is the aldehyde substrate and alcohol product that display the large differential in K_m value.

The relative differential in the use of intrinsic binding energy derived from the oxidized versus the reduced cofactor for *Hs*ADHβ and ALR2 must also relate to the chemistry employed by each enzyme. In the one case, the binary E·nucleotide complexes must overcome the external K_{eq} value, while in the other, the E·nucleotide complexes need merely express K_{eq}. Thus, the ability of the active site Zn^{2+} to coordinate the alkoxide ion during the course of the hydride-transfer reaction undoubtedly contributes to the effectiveness of *Hs*ADHβ as an alcohol dehydrogenase (Cook & Cleland, 1981). Conversely, although the actual residue that serves as a general base has not yet been identified, the data indicate that the ALR2 active site environment will be such as to strongly favor polarization of the carbonyl moiety of the aldehyde substrate and disfavor interaction with the cognate alcohol. Perhaps the best way to demonstrate this reversal is to compare $V/K_{ROH}E_t$ values for ethylene glycol oxidation, since both ALR2 and *Hs*ADHβ will utilize this substrate. Thus, the estimated $V/K_{ROH}E_t$ value for *Hs*ADHβ (400 M^{-1} s^{-1} at pH 8.0[6]) is 80 000-fold greater than the value for ALR2 (cf. Table I), despite the fact that $K_{i\ NAD(P)H}$ and K_{RO} for these two enzymes are within a factor of 4!

Yet, in each case, the binary E·nucleotide complex appears to be "poised" for efficient reaction with a wide range of substrates. These enzymes therefore do not violate the tenet of optimized utilization of intrinsic binding energy but instead circumvent the need for transition-state stabilizing interactions with the second substrate by deriving the necessary energy from the interaction between the enzyme active site and the nucleotide cofactor. The result in either case is a high-efficiency, broad spectrum catalyst.

Superreactive E·Nucleotide Complex. There is good evidence for enhanced reactivity of NADPH when bound at the ALR2 active site. Thus, if one compares the relative log K_{eq} values for nucleophilic addition of sulfur and oxygen nucleophiles and hydride ion to a 2-hydroxyaldehyde, pyridine-4-carboxaldehyde, and *p*-chlorobenzaldehyde, the result is a 3 log difference in susceptibility to attack (Figure 4). However, a similar plot of log $V/K_{RO}E_t$ values for ALR2-catalyzed reduction of the same three aldehydes shows less than a 7-fold variation. In other words, the relative susceptibility of *p*-chlorobenzaldehyde to hydride addition from NADPH bound at the ALR2 active site is enhanced nearly 1000-fold relative to the reaction free in solution.

[6] This is based on the fact that $V/K_{ROH}E_t$ for ethylene glycol is 6% of the value for ethanol at pH 10.0 (Wagner et al., 1983) and the relative ratio of $V/K_{ROH}E_t$ values for primary alcohol substrates remains fairly constant between pH 10.0 and pH 7.5 (Burnell et al., 1989).

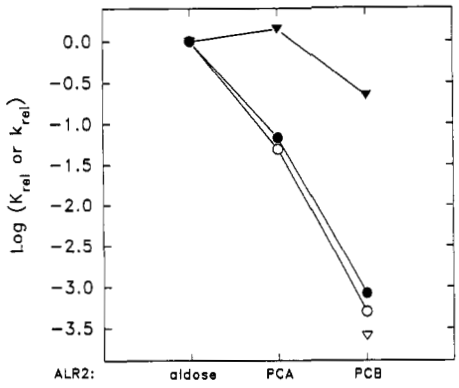
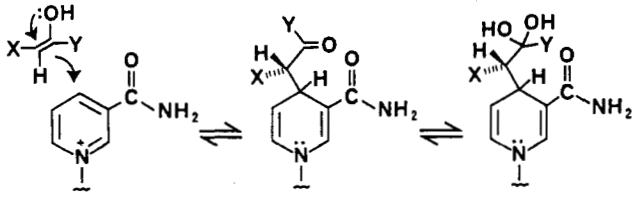


FIGURE 4: Comparison of K_{eq} for nucleophilic addition with catalytic efficiency for ALR2-catalyzed reduction. Relative log K_{eq} values for addition of sulfur (●) and oxygen (○) nucleophiles (Sanders & Jencks, 1968; Grimshaw et al., 1979) and hydride ion (▽) to D-xylose (aldose) (Robinson, 1966; Kornberg & Gotto, 1966; Kormann et al., 1972), pyridine-4-carboxaldehyde (PCA), and *p*-chlorobenzaldehyde (PCB) (Klinman, 1972) are shown vs relative log $V/K_{RO}E_t$ values (Grimshaw et al., 1989) for reduction by ALR2 (▼).

Scheme II[a]



[a] ALR2, X = –OH and Y = –H; LDH, X = –H and Y = –COOH.

A comparison of the energetics for covalent NAD(P)–aldehyde/ketone adduct formation (Scheme II) further suggests that the reactivity of enzyme-bound $NAD(P)^+$ is decreased in ALR2 relative to A_4-LDH. As discussed by Burgner and Ray (1984b), adduct formation is an excellent model for enzyme-catalyzed alcohol oxidation since both reactions utilize the B: form of the enzymic acid–base catalyst, both involve nucleophilic addition to the 4′-position of the nicotinamide ring, and both involve the normal substrates. The free energy diagram shown in Figure 5 summarizes the results of extensive studies of NAD–pyruvate adduct formation catalyzed by dogfish muscle A_4-LDH (Burgner & Ray, 1974, 1978, 1984a,b; Burgner et al., 1978, 1984) and of NADP–glycolaldehyde adduct formation mediated by ALR2 (Grimshaw et al., 1990a,b). As shown, both enzymes significantly enhance the rate and apparent equilibrium position for adduct formation relative to the nonenzymic reaction in aqueous solution. Yet, A_4-LDH displays a much *greater* stabilization of adduct formation at the enzyme active site (solid arrows), with a catalytic advantage (in free energy terms) that is 1.9–3.7 kcal/mol greater than that calculated for ALR2. A_4-LDH shows a somewhat smaller enhancement of the rate of adduct formation (broken arrows) relative to ALR2; however, this may be due to the unusual reactivity of glycolaldehyde.[7] Thus, ALR2 appears to sacrifice a catalytic advantage of between 1.9 and 3.7[8] kcal/mol relative to A_4-LDH in reactivity of the binary $E{\cdot}NAD(P)^+$ complex.

Artificial Evolution. An attempt has been made to generate a broad specificity redox catalyst from a monospecific enzyme

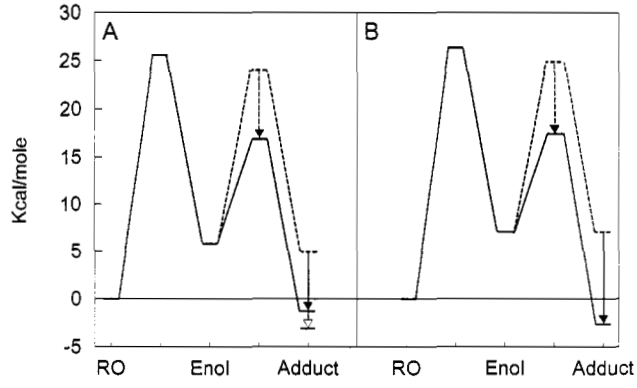


FIGURE 5: Free energy diagram comparing nonenzymic and enzyme-mediated adduct formation for bovine kidney ALR2 and dogfish muscle A_4-LDH. The arrows show $\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{E\text{-adduct}} - \Delta G^{\ddagger}_{adduct}$ for enhancement of the *rate* (broken arrow) of adduct formation at the enzyme active site and $\Delta\Delta G' = \Delta G'_{E\text{-adduct}} - \Delta G'_{adduct}$ for stabilization of the enzyme-bound adduct (solid arrow) produced by reaction of $NADP^+$ with glycolaldehyde enol (ALR2, panel A) or NAD^+ with pyruvate enol (A_4-LDH, panel B), respectively. The data for ALR2 are from Grimshaw et al. (1990a) and for A_4-LDH are adapted from the studies of Burgner, Ray, and co-workers (Burgner & Ray, 1974, 1978, 1984a,b; Burgner et al., 1978, 1984).

via site-directed mutagenesis of specific residues involved in the substrate recognition pocket. In the case of *Bacillus stearothermophilus* lactate dehydrogenase, five separate point mutations were made in the flexible loop and G-helix regions that form the two sides of the pocket which closes around the substrate during catalysis (Wilks et al., 1990). The changes were chosen to mimic the residues found in the broad specificity C_4-isozyme (Hogrefe et al., 1987). Yet, rather than produce an efficient, broad specificity catalyst, the result was instead a "permissive" 2-oxoacid reductase displaying fairly constant $V/K_{RO}E_t$ values for a range of substrates but at a level 2.5 log units *below* the wild-type value for pyruvate reduction.

A similar attempt to increase the catalytic efficiency for oxidation of ethanol by *Sc*ADH1 by substituting a leucine for a methionine residue in the active site was successful and actually produced a catalyst with higher affinity toward long-chain aliphatic alcohols (Ganzhorn et al., 1987). Yet, the $V/K_{ROH}E_t$ values determined for the mutant were still nearly 1 log unit below those of the broad specificity *Sc*ADH2 isozyme and more than 2 log units below those of the *Hs*ADHβ isozyme shown (cf. Figure 3). These results can readily be explained in reference to the new paradigm, since simply making room for bulkier substrates without enhancing the reactivity of the bound nucleotide, whether NAD(P)H or NAD^+, will not reproduce the superreactive, nonselective catalysts seen in ALR2, C_4-LDH, and *Hs*ADHβ.

Physiological Rationale for Broad Specificity Detoxification Enzymes. The pattern that emerges for ALR2 and the other broad specificity enzymes discussed above is that of a high-efficiency catalyst with a low turnover number operating more or less unidirectionally (V/K ratio) to modify a broad range of compounds containing a particular functional group, in this case the aldehyde or ketone moiety. In principle, these kinetic properties could arise if the selection criteria for such broad specificity catalysts were to be flexibility and unidirectionality as opposed to the evolutionary pressure for fluxional efficiency elegantly described by Knowles and co-workers (Albery & Knowles, 1976, 1977; Knowles & Albery, 1977; Burbaum et al., 1989; Burbaum & Knowles, 1989) for monospecific catalysts. Thus, the constraints on a "perfect" detoxification catalyst might be quite different from those on an enzyme optimized for efficient interconversion of a single substrate and product pair. For example, an ideal "aldehyde

[7] ALR2-mediated adduct formation with glyceraldehyde occurs at a slower rate and displays a stereoisomer-dependent change in reaction product that is not seen with glycoaldehyde (Grimshaw, 1991).

[8] The range of free energy values for ALR2-mediated adduct formation derives from the uncertainty as to the extent of hydration of the adduct at the enzyme active site (Grimshaw et al., 1990a).

removease" such as ALR2 should be saturated with cofactor (NADPH) at all times; the extremely low $K_{i\ NADPH}$ and K_{NADPH} values ensure that this is the case. A low $K_{i\ NADP^+}$ value is acceptable provided the physiological ratio of [NADPH]/[$NADP^+$] is such that product inhibition by $NADP^+$ is minimal. Second, the enzyme should have K_m values for aldehyde substrates such that $V/K_{RO}E_t$ remains high for a broad range of substrate types. Such appears to be the case for ALR2. As Jakoby and Ziegler (1990) note, there is a distinct advantage to having detoxification catalysts that are "...specific mainly for functional groups, rather than...one enzyme for each compound".

The rules for an efficient detoxification catalyst conforming to the new paradigm, using ALR2 as the example, can be stated as follows: (1) Maximize binding of the reactive cofactor/cosubstrate (E·NH) to ensure saturation at all times (covalent binding is the extreme case) even if this means that the rate of regeneration of the E·NH complex is rate-limiting for overall turnover. (2) If required, make differential use of the intrinsic binding energy of the reactive (NH) *versus* the spent (N) cofactor to shift the bound-state equilibrium constant as far as possible in the desired direction, up to the point where product inhibition due to formation of the E·N complex becomes significant (which in turn depends on the prevailing [NH]/[N] ratio in vivo).

Rule 1 is apparently the key to maximum utilization of the available intrinsic binding energy of the cofactor/cosubstrate to generate the crucial reactive binary complex. Rule 2 ensures that the enzymic process is optimized to operate within the physiological constraints. Thus, NADPH-dependent aldehyde reduction and NAD^+-dependent alcohol oxidation are ideally suited to the prevailing redox ratios of the two cofactors in vivo, whereas nature has chosen to remove toxic aldehydes using an entirely different NAD^+-dependent process, *oxidation* of the aldehyde by various aldehyde dehydrogenases (Weiner, 1980)!

Notably, all three of the broad specificity catalysts described here have arrived at a similar solution in terms of K_m and V/E_t. Thus, all display k_{cat} values in the 0.2–10-s^{-1} range and K_m values of about 25 μM.[9] Measurement of ALR2 levels in human tissues has shown that muscle contains 5 μM enzyme (Grimshaw & Mathur, 1989). Kidney, which contains only 1 μM ALR2, also contains about 5 μM aldehyde reductase,[10] a closely related NADPH-dependent aldo/keto reductase that has a substrate specificity overlapping that of ALR2. Thus, about 5 μM aldehyde removease is present in each tissue. Given a k_{cat} of 0.2 s^{-1}, this means an effective capacity of 0.5 μM/s for reduction of glyceraldehyde present at 20 μM. Such a capacity is apparently sufficient to ensure the removal of spurious aldehydes that might otherwise react with free amino groups on various proteins (Acharya et al., 1988), on NAD(P) nucleotides (Grimshaw et al., 1990a), or on the adenine and guanine bases in DNA (McGhee & von Hippel, 1975). It may well be that C_4-LDH serves a similar protective function in spermatogenesis in vivo.

Extension to Other Detoxification Catalysts. This new paradigm may well apply to other detoxification enzyme systems. Thus, as noted by Jakoby and Ziegler (1990) in a recent review, certain of the flavin monooxygenases appear to function primarily by stabilizing at the enzyme active site a superreactive hydroperoxyflavin species that is then capable of reacting with essentially any soft electrophile. In the glutathione transferase family, studies by Chen et al. (1988) suggest that the enzyme can enhance the basicity of bound glutathione by lowering the pK_a value nearly 2.5 units without decreasing the nucleophilicity of the thiolate anion. The work of Schowen and co-workers suggests that catechol *O*-methyltransferase, a catechol detoxifying enzyme, achieves high catalytic efficiency (in V/K terms) for reaction with a broad range of catechols via a strong interaction with the cofactor *S*-adenosyl-L-methionine (Olsen et al., 1979). In that case, catalysis is thought to occur via compression of the transition state at the enzyme active site, resulting almost exclusively from interactions with the cofactor and not with the catechol substrate. Recent crystallographic results indicating that large domain movements occur on going from the binary E·2′-phosphoadenosine–5′-diphosphoribose complex (Rondeau et al., 1992) to the binary E·NADPH (Wilson et al., 1992) complex of ALR2 suggest that, analogous to catechol *O*-methyltransferase, it is the strong interaction with the nucleotide cofactor that directly leads to enhanced reactivity (and, thus, low selectivity) of bound NADPH.

The price paid for stabilizing these superreactive species is that overall turnover is often limited by the rate at which the reactive species can be regenerated. The potential detriment to the cell of such a low V/E_t value is apparently more than offset by the ability of a single catalyst to effectively remove and detoxify a broad range of substrate types. Whether this same paradigm can be applied to the sulfotransferases, *N*-methyltransferases, amine oxidases, and other detoxification catalysts and salvage enzymes remains to be established. However, in each case where an efficient, broad specificity, essentially one-way catalyst for detoxification of an entire class of compounds has been required, this need has apparently been met with an enzyme containing a tightly or covalently bound, superreactive (and thus nonselective) cofactor/cosubstrate capable of reacting with virtually any compound meeting the minimal structural criteria.

[9] For this calculation we consider the aldehyde substrate for ALR2 to be the sum of the aldehyde and hydrate.

[10] This is based on the activity determined in bovine kidney (Daly & Mantle, 1982).

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