

REVIEW

Structure and function of yeast alcohol dehydrogenase

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1. Introduction
2. Isoenzymes of YADH
3. Substrate specificity
4. Kinetic mechanism
5. Primary structure
6. The active site
7. Mutations in the yeast enzyme
8. Chemical mechanism
9. Binding of coenzymes
10. Hydride transfer

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Abbreviations: YADH yeast alcohol dehydrogenase, isoenzyme YADH-1; NDMA *p*-nitroso-*N,N*-dimethylaniline; DACA *N,N*-dimethylamino-*trans*-cinnamaldehyde; AA acetamide; Az sodium azide.

1. INTRODUCTION

Yeast alcohol dehydrogenase (EC 1.1.1.1) is a member of a large family of zinc-containing alcohol dehydrogenases. The primary structures of 47 members of this family have been determined and aligned and an evolutionary tree was constructed, assuming a divergent evolution from a common ancestral gene.¹ In this way, it was possible to identify four divergent groups of alcohol dehydrogenases (ADH) in this family: vertebrates, plants, eukaryotic microorganisms and prokaryotic bacteria. Baker's yeast (*Saccharomyces cerevisiae*), a member of the third group, has three isoenzymes of alcohol dehydrogenase: YADH-1, YADH-2 and YADH-3. YADH-1 is the constitutive form that is expressed during anaerobic fermentation.² YADH-2 is another cytoplasmic form which is repressed by glucose³ and YADH-3

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is found in mitochondria.⁴ YADH-1 accounts for the largest part of the alcohol dehydrogenase activity in growing baker's yeast.

The structure, function and mechanism of the action of yeast alcohol dehydrogenase was reviewed two decades ago.^{5,6} The purpose of this article is to update the subject and to review novel data on the structure, function and mechanism of the action of the isoenzyme YADH-1; this isoenzyme will be abbreviated as YADH, throughout the text. Throughout this work, the steady-state kinetic constants are presented using the nomenclature of Cleland.⁷

2. ISOENZYMES OF YADH

Yeast alcohol dehydrogenase was one of the first enzymes to be isolated and purified.⁸ If the steady-state kinetic properties of ADH isoenzymes are compared, a large degree of similarity is detected. Table I shows the steady-state kinetic constants for the three isoenzymes of YADH, isolated from baker's yeast.

TABLE I. Steady-state kinetic constants of yeast ADH isoenzymes with ethanol and acetaldehyde as substrates, at pH 7.3, 30 °C.^a

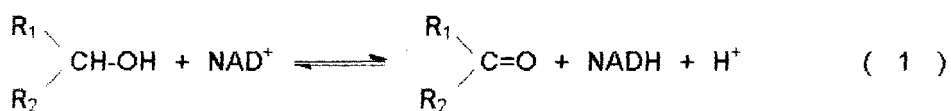
Constant		YADH-1	YADH-2	YADH-3
V_1	s^{-1}	340	130	450
K_A	μM	170	110	240
K_B	mM	17	0.81	12
V_1/K_B	$mM^{-1}s^{-1}$	20	160	37.5
V_2	s^{-1}	1700	1040	2100
K_Q	μM	110	50	70
K_P	mM	1.1	0.09	0.44
V_2/K_P	$mM^{-1}s^{-1}$	1540	11550	4770

^a Calculated from the data of Ganzhorn *et al.*⁹

It is evident that YADH-1 and YADH-3 have very similar kinetic characteristics, while YADH-2 has a much higher substrate specificity for ethanol (V_1/K_B) and acetaldehyde (V_2/K_P), and much lower Michaelis constants with ethanol (K_B) and acetaldehyde (K_P).

3. SUBSTRATE SPECIFICITY

Yeast alcohol dehydrogenase catalyzes the following reversible redox reaction.⁵



At neutral pH the equilibrium is shifted far to the left (Table II).

Substrate specificity of YADH is restricted to primary unbranched aliphatic alcohols, and any branching in the side chain diminishes the activity of enzyme and lowers its efficiency. In addition, the enzyme also shows activity towards secondary alcohols. Table II lists the steady-state kinetic constants for various alcoholic substrates and Table III the steady-state constants for various carbonyl substrates of the yeast enzyme.

TABLE II. Steady-state kinetic constants for the oxidation of various alcohols at neutral pH

Constant		Ethanol ^a	Propan-1-ol ^a	Butan-1-ol ^a	Hexan-1-ol ^b	Decan-1-ol ^b
V_1	s^{-1}	454	67	25	15.4	14.4
K_A	μM	109	150	250	169	200
K_{iA}	μM	325	235	160	152	190
K_B	mM	21.7	29.2	32	3.2	0.1
V_1/K_A	$mM^{-1}s^{-1}$	4165	447	100	91	72
V_1/K_B	$mM^{-1}s^{-1}$	20.9	22.9	0.78	4.8	144
V_1K_{iA}/K_A	s^{-1}	1354	105	16	13.8	13.7
K_{eq} ^f	—	0.00019	—	0.00027	—	—

Constant		Propan-2-ol ^c (<i>S</i>)-(+)-	Butan-2-ol ^c	Allyl alcohol ^d	Ethylene glycol ^d	Tris ^e
V_1	s^{-1}	7	0.9	546	7.0	0.5
K_A	μM	597	376	520	370	698
K_{iA}	μM	378	398	730	550	842
K_B	mM	117	35	14.6	444	6415
V_1/K_A	$mM^{-1}s^{-1}$	11.7	2.4	1058	19.2	0.72
V_1/K_B	$mM^{-1}s^{-1}$	0.06	0.026	37.5	0.016	0.0001
V_1K_{iA}/K_A	s^{-1}	4.4	0.95	766	10.4	0.60
K_{eq} ^f	—	0.146	0.40	—	—	—

Calculated from the data of: ^aDickenson and Monger,¹⁰ at pH 7.0, 25 °C. ^bSchopp and Aurich,¹¹ at pH 8.0, 25 °C. ^cTrivić and Leskovac,¹² at pH 7.0, 25 °C. ^dTrivić and Leskovac,¹³ at pH 7.0, 25 °C. ^eChen and Huang,¹⁴ at pH 8.2, 25 °C. ^f $K_{eq} = V_1K_{iQ}K_P/V_2K_{iA}K_B$.

Ethanol is by far the best substrate of the yeast enzyme. However, allyl and cinnamyl alcohol are also excellent substrates; kinetic constants for the latter alcohol are, $V_1 = 133 s^{-1}$ and $V_1/K_B = 29 mM^{-1}s^{-1}$, at pH 8.2, 25 °C.¹⁷ (*S*)-(+)-Butan-2-ol is a much better substrate than (*R*)-(-)-butan-2-ol ($V_1 = 1.0 s^{-1}$ and $0.05 s^{-1}$, and $V_1/K_B = 18 M^{-1}s^{-1}$ and $0.8 M^{-1}s^{-1}$, respectively, at pH 7.3, 30 °C).¹⁸ 4-Methyl-1-pentanol ($V_1 = 7 s^{-1}$, pH 8.2) is a much better substrate than 2-methyl-1-propanol ($V_1 = 0.2 s^{-1}$, pH 7.3) or 3-methyl-1-butanol ($V_1 = 0.3 s^{-1}$, pH 8.2).^{17,18}

It was reported that glycerol, glyceraldehyde and acetol are poor substrates of YADH¹⁹, benzyl alcohol and benzaldehyde are extremely poor substrates of YADH.^{18,20} It was also reported that *p*-chlorobenzyl alcohol and *p*-methoxybenzyl alcohol are slowly oxidized by NAD^+ in the presence of YADH.²¹ 2-Chloroethanol, 2-fluo-

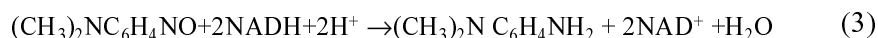
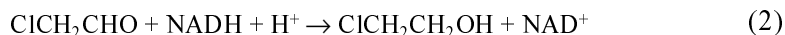
roethanol, 2,2,2-trifluoroethanol, propargyl alcohol, glycidol and poly(ethylene glycol) are definitely *not* substrates of the yeast enzyme.²²

TABLE III. Steady-state kinetic constants for the reduction of various carbonyl substrates at neutral pH

Constant		Acetaldehyde ^a	Butyraldehyde ^a	Acetone ^b	Butan-2-one ^b
V_2	s^{-1}	3850	3450	9	0.7
K_Q	μM	96	97	43	38
K_{iQ}	μM	12.5	7	17.5	15.2
K_P	mM	0.9	27.5	477	285
V_2/K_Q	$mM^{-1}s^{-1}$	40100	35570	209	18.4
V_2/K_P	$mM^{-1}s^{-1}$	4280	125	0.019	0.0025
V_2K_{iQ}/K_Q	s^{-1}	501	249	3.66	0.38
Constant		Chloroacetaldehyde ^c	NDMA ^d	DACA ^e	
V_2	s^{-1}	117	2.1	0.176	
K_Q	μM	270	456	46	
K_{iQ}	μM	74	119	7.6	
K_P	mM	4	1.5	0.61	
V_2/K_Q	$mM^{-1}s^{-1}$	431	4.5	3.8	
V_2/K_P	$mM^{-1}s^{-1}$	25.2	1.4	0.29	
V_2K_{iQ}/K_Q	s^{-1}	31.9	0.54	0.03	

Calculated from the data of: ^a Dickenson and Monger,¹⁰ pH 7.0, 25 °C.; ^b Trivić and Leskovac,¹² pH 7.0, 25 °C.; ^c Leskovac *et al.*,¹⁵ at pH 9.0, 25 °C.; ^d Leskovac *et al.*,¹⁶ at pH 8.9, 25 °C.; ^e Leskovac *et al.*,¹⁵ at pH 7.0, 25 °C.

Yeast alcohol dehydrogenase catalyzes three essentially irreversible chemical reactions:



Chloroacetaldehyde is an excellent substrate of YADH (Table III), while 2-chloroethanol is not oxidized by NAD^+ , which makes the reaction (2) essentially irreversible.¹⁵ NDMA is readily reduced by NADH in the presence of YADH (Eq. 3); the primary product of reaction, the corresponding hydroxylamine, is transformed into a quinonediimine compound by the loss of a molecule of water. The last compound is reduced non-enzymatically by NADH into *p*-amino-*N,N*-dimethylaniline.^{16,23,24}

YADH has a weak aldehyde dehydrogenase activity; it is able to catalyze the irreversible oxidation of acetaldehyde into acetic acid with NAD^+ , with an apparent $k_{cat} = 2.3 s^{-1}$ and $V/K = 34 M^{-1}s^{-1}$, at pH 8.8, 22 °C.²⁵

Free acetaldehyde is a true substrate for the alcohol dehydrogenase,²⁶ and gem-diol is probably a true substrate for the aldehyde dehydrogenase activity of YADH.²⁵

4. KINETIC MECHANISM

Yeast alcohol dehydrogenase catalyzes the chemical reactions described by Eq. (1). Numerous investigations of the steady-state kinetic mechanism of the yeast enzyme were conducted by several authors^{9,10,27-35}; they suggested conclusively that the yeast enzyme follows the *steady-state random* mechanism on the alcohol side, and a *steady-state ordered* mechanism on the aldehyde side of the catalytic cycle, with primary aliphatic alcohols and aldehydes (Scheme 1).



Scheme 1.

The mechanism in Scheme 1 is restricted to primary unbranched aliphatic alcohols and aldehydes, if the latter are present in lower concentrations.³² The initial rate equation for this mechanism, in the forward direction and in the absence of products, is given by equation.³⁶

$$\begin{aligned} \frac{E_0}{V_0} = & \left(\frac{X}{k_9} + \frac{1}{k_5} + \frac{1}{k_7} \right) + \frac{1}{k_1} \left(\frac{Xk_1k_{14}}{k_9k_{13}} + \frac{k_{12} + k_{13}A + k_{11}B}{k_{12} + k_{13}A + (k_{13}/k_1)k_{11}B} \right) \cdot \frac{1}{A} \\ & + \frac{1}{k_3} \left(\frac{Xk_4}{k_9} + \frac{k_{12} + k_{13}A}{k_{12} + k_{13}A + (k_{13}/k_1)k_{11}B} \right) \cdot \frac{1}{B} \\ & + \frac{1}{k_3} \left(\frac{Xk_4}{k_9} + \frac{k_{12} + k_{13}A + k_{11}B}{k_{12} + k_{13}A + (k_{13}/k_1)k_{11}B} \right) \cdot \frac{1}{AB} \end{aligned} \quad (5)$$

where $X = 1 + k_{10}/k_5$

The applicability of Eq. (5) to alcohol oxidation is readily apparent. Equation (5) predicts that the monomolecular kinetic constant V_1 and the bimolecular specificity constants V_1/K_A and V_1/K_B are dependent on the nature of substrate B ; inspection of the data in Table II shows that this is true for all primary unbranched alcohols. Also, Eq. (5) predicts that the inhibition constant K_{iA} is dependent on the nature of substrate B and, therefore, cannot be equal to the dissociation constant of the $E \cdot NAD^+$ complex; Table II shows that this is true for all the above alcohols. In

addition, the direct determination of $K_{E \cdot NAD^+}$ shows that it is not equal to K_{iA} , in any case (Fig. 3).

The initial rate equation in the reverse direction, reduction of aldehydes, and in the absence of substrates of the reaction, is given by the general expression for the steady-state ordered mechanism:³⁷

$$\frac{E_0}{V_0} = \left(\frac{Y}{k_2} + \frac{1}{k_4} + \frac{1}{k_{10}} \right) + \frac{1}{k_8 Q} + \frac{Y k_5 + k_{10}}{k_8 k_{10} P} + \frac{k_7 (Y k_5 + k_{10})}{k_8 k_9 k_{10} P Q} \quad (6)$$

where $Y = 1 + k_9/k_4$.

Equation (6) satisfies the results obtained for the reduction of acetaldehyde and butyraldehyde in predicting a linear reciprocal equation, in which the K_{iQ} , V_2/K_Q and $V_2/K_{iQ}/K_Q$ constants are independent of the nature of the aldehyde (Table III).

The kinetic mechanism presented in Scheme 1 is compatible with the deuterium isotope effects on maximal rates reported for ethanol, $^D V_1 = 1.8$, $^D V_1/K_A = 1.8$, and $^D V_1/K_B = 3.2$,³⁸ propan-1-ol, $^D V_1 = 3.7$,³⁹ butan-1-ol, $^D V_1 = 3.740$ and propan-2-ol, $^D V_1 = 2.2$,¹² around neutrality. With ethanol, the effect on $^D V_1/K_A$ was smaller than on $^D V_1/K_B$, suggesting that NAD^+ binds before ethanol; the still significant size of $^D V_1/K_A$ is probably due to dissociation of NAD^+ from the ternary complex.³⁸

With propan-2-ol and acetone, the kinetic mechanism is steady-state random in both directions.¹² A similar kinetic mechanism probably holds for most branched and secondary alcohols.³³

The magnitudes of the individual rate constants in Scheme 1 were calculated from reaction progress curves, in both directions, with the aid of KINSIM and FITSIM computer programs^{41–43} (Table IV).

TABLE IV. Rate constants for the mechanism in Scheme 1, with ethanol and acetaldehyde as substrates, at pH 7.0, 25 °C⁴⁴

k_1	$\mu M^{-1} s^{-1}$	7.0	k_7	s^{-1}	388
k_2	s^{-1}	2100	k_8	$\mu M^{-1} s^{-1}$	28.5
k_3	$\mu M^{-1} s^{-1}$	1.72	k_9	s^{-1}	4000
k_4	s^{-1}	273 000	k_{10}	s^{-1}	35000
k_5	s^{-1}	11000			
k_6	$\mu M^{-1} s^{-1}$	5.0			

^aThe concentrations of reactants were chosen in such a way, as to prevent the dissociation of NAD^+ from the EAB complex, and thus exclude the rate constants k_{11} – k_{14} .

An extremely high value obtained for the rate constant k_4 in Table IV indicates that, in the reverse direction, alcohol dissociates very rapidly from the EAB complex. An alternative explanation is that both constants, k_3 and k_4 , are composite constants, including the rate constants for the isomerization of the EA complex. However, direct experimental proof for the isomerization step in the yeast enzyme

is still missing.^{31,38} In the horse liver enzyme, when the coenzyme binds, it triggers a large change in the conformation of the enzyme, well documented both in structural terms⁴⁵ and by kinetic methods.⁴⁶

Inspection of the data in Table IV clearly shows that, in the forward direction, oxidation of ethanol at neutral pH, the rate-limiting step is not the chemical reaction (k_9), but the dissociation of NADH from the EQ complex (k_7). Likewise, NAD^+ dissociates much faster from the EA complex (k_2) than NADH dissociates from the EQ complex (k_7).

5. PRIMARY STRUCTURE

YADH-1 is a tetramer, composed of four identical subunits; each subunit is made of a single polypeptide chain with 347 amino acids, with a molecular weight of 36000.⁴⁵ Each subunit has one coenzyme-binding site and one firmly bound zinc atom, which is essential for catalysis.⁴⁷ The catalytic domain provides the ligands for the catalytic zinc atom: Cys-46, His-67 and Cys-174. The second zinc atom/subunit is liganded in a tetrahedral arrangement by four sulfur atoms from cysteine residues 97, 100, 103 and 111; this zinc atom has only a structural role.⁴⁸

Table V shows the primary structures of the three isoenzymes of YADH.^{4,49-51} The alignment of the amino acid residues for all 47 members of the ADH family was made progressively rather than pairwise.¹

TABLE V. Primary structure of the three isoenzymes of yeast alcohol dehydrogenase

	10	20	30
YADH-1	S I PETQK	GV I FYESHGK	LEYKD I PVPK
YADH-2	S I PETQK	AI I FYESNGK	LEHKD I PVPK
YADH-3	QSTAA I PKTQK	GV I FYENKGK	LHYKD I PVPE
40	50	60	70
PKANELL I NV	KYSGVCHTDL	HAWHGDWPLPVK	LPLVGGHEGA
PKANELL I NV	KYSGVCHTDL	HAWHGDWPLPVK	LPLVGGHEGA
PKANELL I NV	KYSGVCHTDL	HAWHGDWPLPVK	LPLVGGHEGA
80	90	100	110
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
111	120	130	140
C*****	*****PHA	DLSG*****Y	THDGSFQQYA
C*****	*****PHA	DLSG*****Y	THDGSFQQYA
C*****	*****PHA	DLSG*****Y	THDGSFQQYA
160	170	180	190
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN

TABLE V. contd.

200	210		
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
240	250	260	270
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
280	290	300	310
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
320	330	340	350
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMGENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
GVVVGMCENV	KGWKIGDYAG	IWLNGSCMAC	EYCELGNESN
360	370		
**STLPEIYE	KMEKGQ I VGRY	VVDTSK	
**SSLPEIYE	KMEKGQ I AGRY	VVDTSK	
**SELPKVYD	LMEKGK I LGRY	VVDTSK	

The numbering of amino acids corresponds to horse liver alcohol dehydrogenase; alignment and numeration of amino acid residues according to Sun and Plapp.¹

As a result, the primary structures of yeast ADH isoenzymes have some apparent deletions or insertions at several positions. A large deletion is found for residues 112–127 and residues 135–139, which are present in the horse liver enzyme but are absent in the yeast enzyme. This segment forms a surface loop in the liver enzyme subunit far from the active site.

If the primary structures of ADH isoenzymes from baker's yeast are compared, a large degree of homology and similarity is detected. YADH-2 shows 80% and YADH-3 90% structural homology with YADH-1. Evidently, all three isoenzymes display a high degree of structural homology, which is especially visible if the catalytically important residues are compared.

6. THE ACTIVE SITE

Amino acid sequences of horse liver alcohol dehydrogenase and YADH-1 are homologous, and the homology amounts to 25 % amino acid residues.⁵ YADH-1 has been crystallized, but only preliminary crystallographic studies were reported.⁵² The three-dimensional structure of horse liver alcohol dehydrogenase in several binary and ternary complexes with coenzymes, substrates and inhibitors has been

solved at high resolution.⁴⁵ The tertiary structures of the liver and yeast enzyme are highly similar and able to accommodate the extensive sequence changes between the enzymes.⁵³

By analogy with the liver enzyme, the subunits of the yeast enzyme are probably divided into two domains: the catalytic and the coenzyme-binding domain. The two domains are unequal in size; the catalytic domain contains 3/5 of all amino acids, whereas the coenzyme-binding domain contains the remaining 2/5 of the amino acids. The domains are separated by a cleft containing a deep pocket which accommodates the substrate and the nicotinamide moiety of the coenzyme. One domain binds the coenzyme and the other provides ligands to the catalytic zinc, as well as to most of the groups that control substrate specificity.⁴⁵

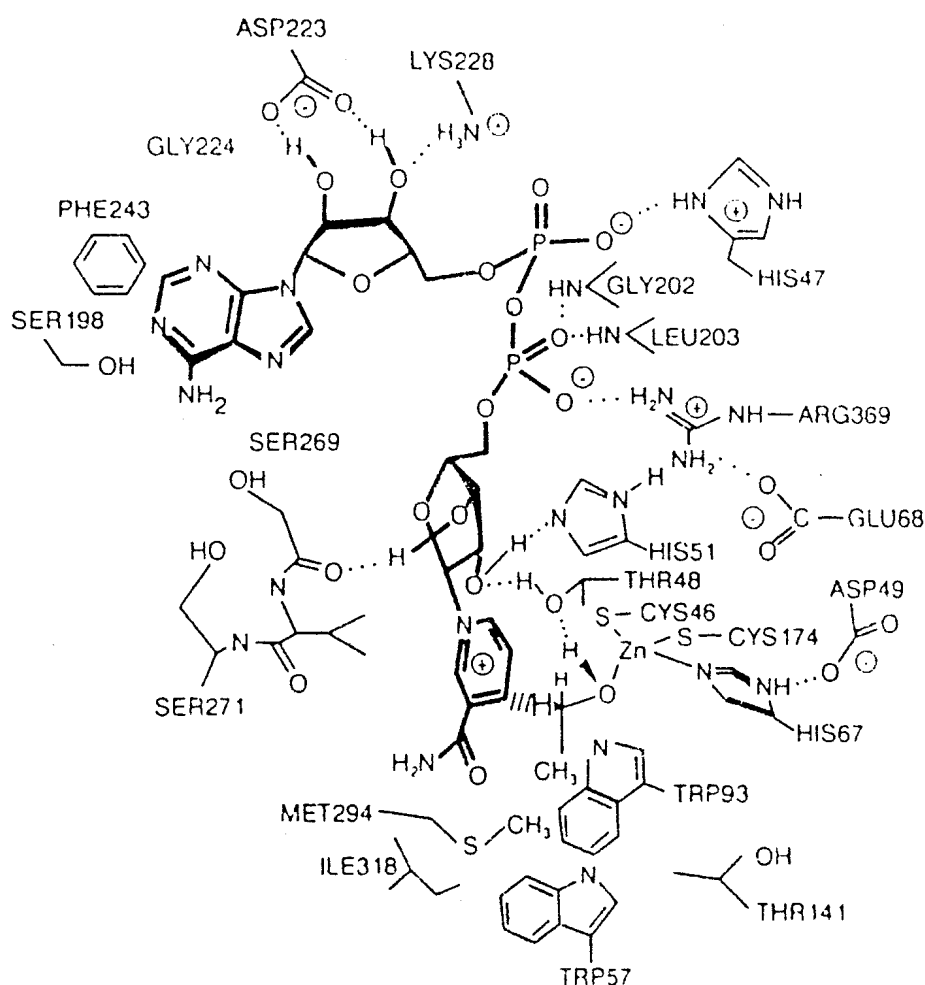


Fig. 1. Model of the active site of yeast alcohol dehydrogenase.⁵⁴

Since the liver and yeast enzymes are homologous, molecular modeling of the yeast enzyme can approximate the structure of one subunit, but not yet the quaternary arrangement.⁵³

Figure 1 shows a model of the active site of the yeast enzyme, obtained in a molecular graphics display system.⁵⁴ The 3D-model of the active site of YADH provides an illustration of the main working machinery of the yeast enzyme.

In order to perform catalysis, the active site of the enzyme must bind a molecule of substrate and a molecule of coenzyme in a productive mode, and catalyze a hydride transfer reaction between them. Numerous amino acid residues in the primary structure of enzyme are involved in this task (Table VI).

TABLE VI. Positions of residues that participate in the enzymatic functions of the yeast enzyme (adopted from Jornval *et al.*⁵³)

<i>Adenine binding pocket</i>		Nicotinamide	
<i>Interior</i>		Thr-178	
Ser-198	Gly-250	<i>Substrate binding pocket</i>	
Ile-222	Ala-274		
Gly-224	Ala-277		
Phe-243			
<i>Surface</i>		Trp-93	Met-294
Ser-271	Ala-273	Asn-110	Ala-296
		Leu-132	Tyr-318
		Tyr-140	
<i>Adenosine ribose binding</i>		<i>Proton relay system</i>	
Gly-199	Lys-228	Thr-48	His-51
Asp-223	Ser-269		
Gly-225			
<i>Pyrophosphate binding</i>		<i>Ligands to active site zinc atom</i>	
		Inner sphere	
		Cys-46	Cys-174
His-47	Leu-203	His-67	
Gly-202	Arg-369		
<i>Nicotinamide ribose</i>		Second sphere	
		Asp-49	Glu-68
Gly-293			

The *adenosine-binding site* is easily accessible from solution, while the nicotinamide binding site is at the center of the molecule, buried deep inside the protein.⁴⁵

Substrate binding pocket. The inside wall of the pocket is lined with hydrophobic side chains from residues Trp-57, Trp-93, Asn-110, Leu-132, Tyr-140, Thr-141, Met-294, Ala-296, and Tyr-318, which are from the same subunit as the zinc ligands. The substrate binding site near the zinc is narrow, because Trp-93 and

Thr-141 limit access. The substrate binding pocket in the yeast enzyme is much narrower than the corresponding pocket in the equine liver enzyme, because it contains voluminous side chains of the amino acids Trp-57, Trp-93 and Met-294.

Ligands to the active site zinc. At the bottom of the substrate binding pocket, a Zn atom is coordinated to three protein ligands: two thiolates from Cys-46 and Cys-174, and an imidazole nitrogen from His-67; the other imidazole nitrogen of His-67 is hydrogen-bonded to the carboxylic group of Asp-49. The carboxylic group of Glu-68 is also in close proximity to the active site zinc atom. Asp-49 and Glu-68 are the residues that are conserved in all known Zn-dependent alcohol dehydrogenases; both amino acids are not innersphere ligands to the zinc, but are in the second sphere. The only polar group in the pocket close to the zinc are its ligands, the nicotinamide moiety of the coenzyme, and the side chain of Thr-48.

Nicotinamide ring. The nicotinamide ring binds in a cleft in the interior of the protein, close to the center of the molecule. The ring interacts on one side with Thr-178, Leu-203 and Met-294. The other face is directed towards the active site, and is close to the catalytic zinc atom and the sulfur ligands of Cys-46 and Cys-174. The oxygen atom of the carboxamide is hydrogen bonded to the main chain nitrogen atom of Val-319. The nitrogen atom of the carboxamide group is hydrogen bonded to the carboxyl oxygens of Val-292 and Ser-317. The side chain of Thr-178 helps to keep the nicotinamide ring of the nucleotide in a correct stereochemical position for hydride transfer (Fig. 4); Thr-178 is conserved in all known homologous alcohol dehydrogenases.

The proton relay system. It was proposed⁵⁵ that the hydrogen-bonded relay system in the liver enzyme, His-51....NAD⁺....Ser-48....RCH₂OH(H₂O)....Zn²⁺, stretching from His-51 on the surface of the enzyme to the active site zinc atom in the interior of enzyme, serves as a proton conductor that helps the dissociation of alcohol to alcoholate in the productive ternary complex enzyme NAD⁺ alcohol. The yeast enzyme has the same proton relay system with, however, Ser substituted with Thr⁵⁴ (Fig. 1). Therefore, since the enzyme NAD⁺.alcoholate complex is considered a true transition state in yeast enzyme catalysis,⁵⁶ the proton relay system must greatly accelerate the same.

Binding of the coenzyme. The coenzyme is bound to the apoenzyme by numerous secondary valence forces. Important amino acid residues are: Asp-223, which is hydrogen-bonded to AMP ribose, His-47, forming a salt bridge with AMP orthophosphate, and Leu-203, forming a hydrogen-bond to NMN orthophosphate.

7. MUTATIONS IN THE YEAST ENZYME

The four yeast ADH genes have been cloned and described.^{4,50,51} Therefore, it was possible to change individual amino acids in the primary structure of YADH-1 by site-directed mutagenesis, and isolate the mutated enzymes in quantity. In recent years, a number of genetically engineered mutants of YADH-1 have been isolated and kinetically characterized, principally by Plapp and his coworkers. Most of these

mutations involve amino acids which are intimately involved in the binding of substrates and in the catalysis, and provide information about the general principles concerning the function of the residues in catalysis. Table VII shows the steady-state kinetic properties of all YADH mutants described so far in the literature.

TABLE VII. Steady-state kinetic constants for YADH mutants, with ethanol and acetaldehyde as substrates, determined at pH 7.3, 30 °C

Mutant	V_1 (s ⁻¹)	V_1/K_A (mM ⁻¹ s ⁻¹)	V_1/K_B (mM ⁻¹ s ⁻¹)	V_2 (s ⁻¹)	V_2/K_Q (mM ⁻¹ s ⁻¹)	V_2/K_P (mM ⁻¹ s ⁻¹)	Reference
YADH-1	340	2000	20	1700	15500	1545	9
Met294Leu	500	794	26.3	2100	26250	2100	9
Trp57Met	220	265	4.9	1900	6790	513	18
Trp57Leu*	99	91	7.4	211	2245	ND	17
Trp93Ala	110	48	0.07	ND	ND	ND	18
Asp49Asn	7.5	0.83	0.02	113	125	2.3	38
Glu68Gln	9.9	24	0.24	730	4560	13	38
Thr48Ser	200	2200	11.8	1500	13640	2027	18
Thr48Ser: Trp93Ala	140	152	0.033	530	4077	5.7	18
Thr48Ser: Trp57Met: Trp93Ala	120	22.2	0.75	ND	ND	ND	18
Thr48Cys	<1	No detectable activity					54
Thr48Ala	<1	No detectable activity					54
His51Gln	27	245	1.4	2800	25450	215	57
His51Glu	2	26	0.26	ND	ND	ND	54
His47Arg	60	400	0.9	460	46000	98	31
Leu203Ala*	106	56.4	ND	ND	ND	ND	58
Leu203Ala: Thr178Ser*	31.9	61.3	ND	ND	ND	ND	58
Asp223Gly	38	2.1	0.2	300	60	75	59

ND = Not determined; *Determined at pH 8.2, 25 °C.

Substrate binding pocket (Met-294, Trp-57, Trp-93). An exchange of Leu for Met-294, on the edge of the substrate binding pocket, has very little influence on the steady-state kinetic properties of enzyme with ethanol or acetaldehyde. On the other hand, the Met294Leu mutant has a 10-fold lower catalytic activity (V_1) with butan-1-ol, indicating that the C4-atom of butan-1-ol is in close contact with Met294, while the shorter ethanol is not.⁹ An exchange of Met or Leu for Trp-57 decreases the catalytic efficiency (V_1/K_B) with ethanol only 3-4fold, while an exchange of Ala for Trp-93 decreases the catalytic efficiency 300 times. In the latter case the substrate binding

pocket is enlarged and the enzyme acquires weak activity with branched chain alcohols (2-methyl-1-butanol, 3-methyl-1-butanol) and benzyl alcohol.^{17,18}

Ligands to the active site zinc (Asp-49, Glu-68). The carboxylate group of Asp-49 is hydrogen-bonded to His-67, which in turn coordinates the active site zinc; in addition, the carboxylate group of Glu-68 is in the vicinity of the active site. If Asn is substituted for Asp-49 or Gln for Glu-68, a negative charge is removed from the vicinity of the active site zinc; these substitutions reduce the catalytic efficiency with ethanol (V_1/K_B) 1000 and 100 times, respectively, and the catalytic constant (V_1) 40 times. These reductions in activity were interpreted by an increased electrostatic potential near the active site zinc, due to the removal of negative charges; this change decreases the activity by hindering isomerizations of the enzyme-substrate complexes.³⁸

The proton relay system (Thr-48, His-51). An exchange of Ser for Thr-48 does not interrupt the hydrogen bonding in the proton relay system and, as expected, the activity of the Thr48Ser mutant is very similar to that of the wild type. The double mutant Thr48Ser:Trp93Ala and the triple mutant Thr48Ser:Trp57Met:Trp93Ala, have decreased activities which are obviously due to the removal of the bulky tryptophan residues from the substrate binding pocket.¹⁸ An exchange of Cys or Ala for Thr-48 disrupts the hydrogen bonding in the relay system and, as expected, renders the enzyme inactive.⁵⁴

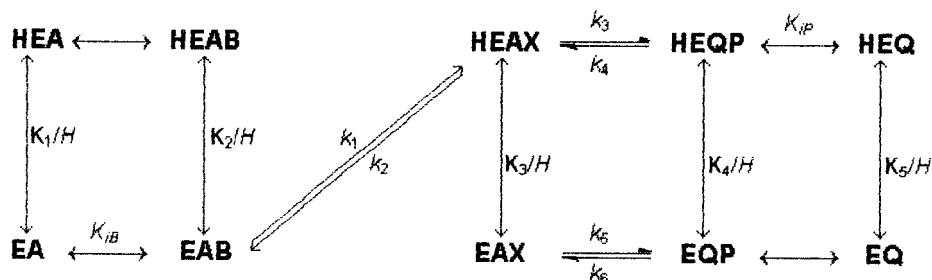
The role of His-51 in catalysis was tested by replacing it with glutamine or glutamic acid.^{54,57} These residues have the appropriate size to form a hydrogen bond with the 2'-hydroxyl group of the nicotinamide ribose; thus, the binding of the coenzyme in the mutant enzymes could resemble that found in the wild enzyme. On the other hand, a glutamine residue cannot participate in base catalysis, whereas a glutamate can accept a proton. Plapp *et al.*⁵⁴ have found that the wild enzyme has a distinct pK_a value of 7.7 in the pH-profile for the V_1/K_B function. Replacement of His-51 with Gln or Glu reduces the value of V_1/K_B 13-fold and 60-fold at pH 7.3, respectively. In addition, the pK_a value of 7.7 in the pH profile of V_1/K_B function is absent in both cases. These results were interpreted by a mechanism in which the amino acid residue in the mutant enzyme hinders the deprotonation of the alcohol through the proton relay system.⁵⁴ These results are consistent with this interpretation, and the role of His-51 in the proton relay system, where it participates as a base.

Binding of the coenzyme (His-47, Leu-203, Thr-178, Asp-223). Exchange of His-47 with Arg does not produce any dramatic change in catalytic properties of the enzyme. Mutations of amino acids Leu-203, Thr-178 and Asp-223 were performed in order to locate the structural determinants of the high stereospecificity of the enzyme.^{58,59}

8. CHEMICAL MECHANISM

The primary and tertiary structure, as well as the point mutations in the yeast enzyme, outlined in the preceding sections, strongly suggest that the integrity of the proton relay system is indispensable for the activity of the enzyme. This integrity

of the relay system, which is maintained throughout the catalytic cycle, led Cook and Cleland,⁵⁶ to propose the following chemical mechanism of action of the yeast enzyme (Scheme 2):



Scheme 2.

In this mechanism, B and P represent the alcohol and ketone, and k_3 , k_4 , k_5 and k_6 represent the hydride-transfer steps; X is an intermediate with the stoichiometry of an alkoxide, and k_1 and k_2 are the steps in which a proton is transferred from B to a group on the enzyme to give X, and similarly for the reverse process.

An assignment of the appropriate pK_a values to all the dissociation forms of the enzyme in Scheme 2 was founded on studies of the pH-dependence of steady-state kinetic and ligand binding parameters,^{12,15,32–35,44,60} as outlined below.

Table VIII shows the macroscopic pK_a values calculated from the pH-profiles of the maximal rates (V_1) and the specificity constants (V/K) with various substrates of the enzyme. Table IX shows the pK_a values calculated from the pH-profiles of binding constants (K_i) for competitive dead-end inhibitors.

TABLE VIII. Macroscopic pK_a values and pH-independent limiting constants in various YADH-catalyzed reactions (adopted from Leskovac *et al.*³⁵)

Substrate	pK_a	Limiting constant	Function	Substrate	pK_a	Limiting constant	Function
Butan-1-ol V_1 (s^{-1})	6.1 7.3 8.3	191	increases with pH	Acetone V_2/K_P ($M^{-1}s^{-1}$)	7.9 8.2 9.0	6.9	decreases with pH
Propan-2-ol V_1 (s^{-1})	6.2 7.4 8.3	81	increases with pH	DACA V_2/K_P ($mM^{-1}s^{-1}$)	8.0	0.25	decreases with pH
Propan-1-ol V_1/K_B ($mM^{-1}s^{-1}$)	6.7 7.4 8.2	9.0	increases with pH	NDMA ^a V_2/K_P ($mM^{-1}s^{-1}$)	8.0	2.2 0.9	plateau at low pH plateau at high pH
Propan-2-ol V_1/K_B ($M^{-1}s^{-1}$)	6.5 7.1 7.8	155	increases with pH				

^a Calculated from the data of Leskovac *et al.*¹⁶

The specificity constants V/K with "nonsticky" substrates, such as propan-1-ol, propan-2-ol, NDMA, DACA and acetone, provide information on the catalytically active groups in the enzyme-coenzyme complexes,⁶¹ if the pH-profiles of V/K are fitted to the initial rate equations appropriate to the mechanism in Scheme 2.³⁵ In this way, the pK_1 (8.0) and pK_5 (7.9–8.0) values in Scheme 2 were estimated. From the binding of azide, a dead-end inhibitor competitive with alcohols, the value for pK_1 (7.9) was confirmed; from the binding of acetamide, a dead-end inhibitor competitive with aldehydes, the values for pK_4 (8.3) and pK_5 (7.9) were estimated.

TABLE IX. Macroscopic pK_a values and pH-independent constants for ternary complexes of YADH with competitive dead-end inhibitors^a

Complex	pK_a	Limiting constant
$E \cdot NAD^+ + Az \rightleftharpoons E \cdot NAD^+ \cdot Az$	7.9	0.95 mM (at low pH)
$E \cdot NADH + AA \rightleftharpoons E \cdot NADH \cdot AA$	8.3	45.8 mM (low pH)
		118 mM (high pH)

^a Calculated from the data of Leskovac *et al.*³⁴

The pH profiles for the V_1 function provide information on the catalytically active groups in the productive ternary complex enzyme·NAD⁺·alcohol.⁶¹ In this way, from the pH-profiles of V_1 with butan-1-ol and propan-2-ol, the pK_2 value was estimated (8.3).

An indirect estimation provided the value of pK_3 (8.3).³⁵

The chemical mechanism of action, shown in Scheme 2, can be drawn entirely in terms of the proton relay system as it is shown in Fig. 2 with, however, Thr-48 omitted for the sake of simplicity. The key feature of Fig. 2 is that His-51 lies at the surface of the protein and thus can be deprotonated as in the conversion of HEAX to EAX or HEQP to EPQ, while the reactants are bound and the state of protonation of molecules in the substrate binding site is locked. Thus, HEAX can be deprotonated to EAX without preventing subsequent hydride transfer.

9. BINDING OF COENZYMES

The binding of coenzymes to apoenzyme are not included in Scheme 2; Figure 3 summarizes the data for the binding of coenzymes to the free enzyme.

The dissociation constant of the $E \cdot NAD^+$ complex for the yeast enzyme, $K_{E \cdot NAD^+}$, is practically pH-independent; on the other hand, the dissociation constant of the $E \cdot NADH$ complex, $K_{E \cdot NADH}$, decreases in the acid over three apparent pK_a values (6.6, 8.0, 9.0). The association rate constant for the binding of NADH to free enzyme (k_8) decreases in alkaline over a single pK_a value 7.8, while the dissociation rate constant for the $E \cdot NADH$ complex (k_7) is almost pH-independent, from pH 6.5–9.0.

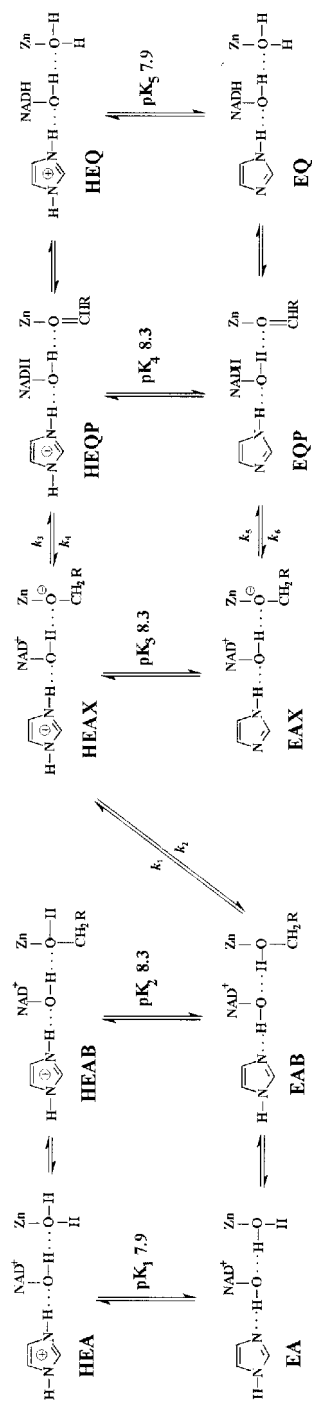


Fig. 2. The chemical mechanism of action of yeast alcohol dehydrogenase.

10. HYDRIDE TRANSFER

One of the classical aspects of coenzyme binding to yeast alcohol dehydrogenase is the A stereospecificity of the coenzyme.⁶² YADH-catalyzed reactions are highly stereospecific; the enzyme catalyzes the transfer of the *Re* hydrogen (pro-*R* or A type) at the 4 position of NADH to the carbonyl carbon of the substrate (Fig. 4). The stereochemical fidelity of the hydride transfer reaction is very high, and YADH makes one stereochemical "mistake" every 7000000 turnovers.

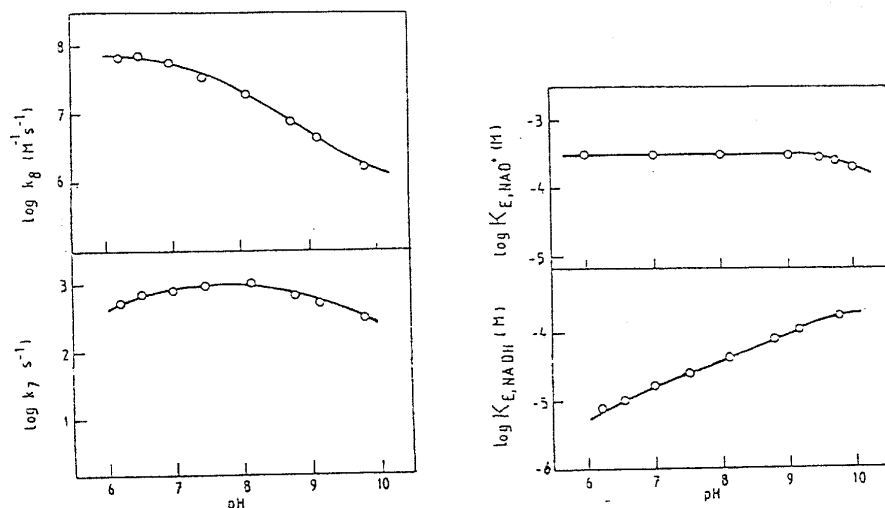


Fig. 3. pH-Profiles for the binding parameters of coenzymes to the free enzyme; rate constants k_7 and k_8 , as in Scheme 1 (adopted from Leskovac *et al.*³³).

If the bulky side chain of Leu-203 is exchanged with Ala, the Leu203Ala mutant (Table VII) makes one stereochemical "mistake" every 850000 turnovers with NADH, and every 450 turnovers with thio-NADH, which has a weaker hydrogen bonding capacity. From this, it was concluded that the decrease in stereochemical fidelity comes from an increase in the rate of transfer of the 4-*Si* hydrogen of NADH. The nicotinamide ring of the coenzyme is kept in the correct position for hydride transfer mainly by hydrogen bonds between its amide group and Val-292 and Val-319, and the rotation of 180° around the glycosidic bond is obstructed mainly by the side chain of Leu-203.⁵⁸

The main reaction catalyzed by alcohol dehydrogenase is, in principle, a very simple reaction. An alcohol group is oxidized by the removal of a proton from the hydroxyl group and by the transfer of a hydride ion from the adjacent carbon atom to NAD⁺. By analogy with the horse liver enzyme,⁴⁵ it may be assumed that hydride transfer in the yeast enzyme occurs in a completely water-free environment. Direct transfer of a hydride ion is facilitated in a hydrophobic environment where water is excluded. The positive charge on the nicotinamide ring is crucial for the enhanced binding of alcohol to the enzyme; the insertion of the positive charge in this hydrophobic environment facilitates formation of the negatively charged alcoholate ion.

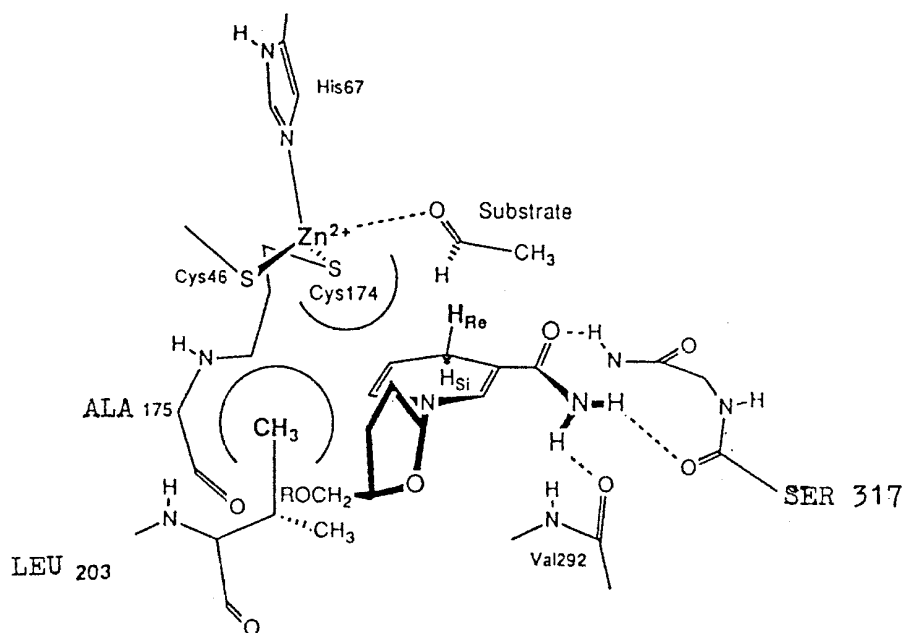


Fig. 4. Stereospecificity of YADH-catalysis. NADH binds *anti*, presenting *Re* hydrogen (H_{Re}) to acetaldehyde lying above the coenzyme in this diagram. For clarity, Thr-178 is not shown; the methyl group of this side chain lies below and to the left of the nicotinamide behind Leu-203 (adopted from Weinhold *et al.*⁵⁸).

The creation of an alcoholate ion greatly facilitates hydride transfer. The important role of the zinc atom in alcohol oxidation is to stabilize the alcoholate ion for the hydride transfer step. In the reverse direction, zinc has the role of electron attractor, which gives rise to an increased electrophilic character of the aldehyde, consequently facilitating the transfer of a hydride ion to the aldehyde. Thus, the proposed mechanism is essentially electrophilic catalysis mediated by the active site zinc atom.

The overall oxidation of alcohol to aldehyde involves a net release of one proton (Eq. 1); the ultimate source of this proton is the alcohol. The release of proton from the bound alcohol occurs in the center of the enzyme molecule in a region that is inaccessible to solution; the proton is transferred by groups on the enzyme to the surrounding solution (Fig. 2). Water is not a component of the reaction. Because water is not involved directly in the catalytic reaction, *i.e.*, there is no hydrolysis or hydration, there is no necessity to suggest a role for a water molecule at the active site of YADH.

In the catalysis, the molecules of substrate and the nicotinamide ring of the coenzyme probably do not have fixed positions. The rearrangement of the electron configuration on the carbon atom from sp^2 hybridization in the aldehyde to sp^3 in the alcohol, requires different pathways for hydride transfer and, consequently, different relative orientations.⁶³

Primary and secondary kinetic isotope effects (k_H/k_D , k_H/k_T and k_D/k_T) in YADH-catalyzed reactions have been studied as a probe of the quantum mechanical hydrogen tunneling. A moderate degree of hydrogen tunneling has been confirmed in the hydride-transfer reactions between benzyl alcohol and NAD^+ ,⁶⁴ and propan-2-ol and NAD^+ .⁶⁵ For hydrogen tunneling to occur, the reactive carbons must be brought close together so that the classical energy barrier is penetrated.⁶⁶ Thus it appears that hydrogen tunneling is an additional general phenomenon which facilitates the YADH-catalysis.

SUMMARY

The structure, function and mechanism of action of yeast alcohol dehydrogenase was reviewed two decades ago. The purpose of this article is to update the subject and to review novel data on the structure and function of the isoenzyme-1 (constitutive, cytoplasmic) of yeast alcohol dehydrogenase, a terminal enzyme in alcoholic fermentation. In this review, a special attention has been paid to the chemical mechanism of action of the enzyme which has been solved recently. In addition to structural and kinetic investigations, the chemical mechanism of action of the enzyme was supported by the site-directed mutagenesis of the yeast alcohol dehydrogenase genes, and investigations of the kinetic properties of numerous mutated enzymes. The comparison of the chemical mechanism of action of the yeast and equine liver alcohol dehydrogenase revealed numerous differences between the microbial and the mammalian enzyme, especially in the binding of coenzymes.

ИЗВОД

СТРУКТУРА И ФУНКЦИЈА АЛКОХОЛ ДЕХИДРОГЕНАЗЕ КВАСЦА

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Ревиије о структури, функцији и механизму дејства алкохол дехидрогеназе квасца су објављене пре више од двадесет година. У овој ревији су изнесени нови подаци о структури и функцији изоензима-1 (конститутивни, цитоплазматски) алкохол дехидрогеназе квасца, терминалног ензима алкохолног врења. Посебна пажња је поклоњена хемијском механизму дејства ензима алкохолног врења, чији механизам дејства је недавно дешифрован. Поред структурних и кинетичких радова, хемијски механизам дејства ензима је такође подупрт мутацијама гена алкохол дехидрогеназе и истраживањем кинетичких особина мутираних ензима. Упоређивањем хемијског механизма дејства алкохол дехидрогеназе квасца са истим ензимом изолованим из јетре коња, утврђене су многобројне разлике између ензима једног сисара и једног микроорганизма, нарочито у везивању коензима.

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