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Binding of coenzymes to yeast alcohol dehydrogenase

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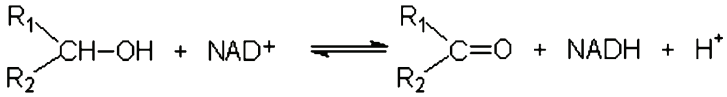
Abstract: In this work, the binding of coenzymes to yeast alcohol dehydrogenase (EC 1.1.1.1) were investigated. The main criterions were the change in the standard free energies for individual reaction steps, the internal equilibrium constants and the overall changes in the reaction free energies. The calculations were performed for the wild type enzyme at pH 6–9 and for 15 different mutant type enzymes, with single or double point mutations, at pH 7.3. The abundance of theoretical and experimental data enabled the binding of coenzymes to enzyme to be assessed in depth.

Keywords: coenzyme binding; Gibbs free energy; yeast alcohol dehydrogenase.

INTRODUCTION

Yeast alcohol dehydrogenase (EC 1.1.1.1, isoenzyme I) catalyzes the oxidation of primary and secondary alcohols (B) by NAD^+ (A) into the corresponding aldehydes or ketones (P) and NADH (Q):



(1)

Reaction (1) is fully reversible and the equilibrium is shifted far to the left at neutral pH.[1]

In this paper, the investigation of coenzyme binding in the above reactions is reported, using the changes in the standard free energies as the main criterion of enzyme thermodynamics. The change in the standard free energies was calculated for individual reaction steps, the internal equilibrium constants in the reaction and the overall changes in the reaction free energies. The calculations were performed for the wild type enzyme at different pH values, mostly from pH 6–9,

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and for mutant enzymes at pH 7.3. An abundance of theoretical and experimental data enabled the coenzyme binding to be investigated in depth.

In addition, the changes in the standard enthalpies and standard entropies of the reaction were calculated for the binding of coenzymes to the free enzyme.

METHODS

The standard transformed Gibbs energies of enzyme-catalyzed reactions, $\Delta_r G°'$, are connected with apparent equilibrium constants of reactions, K', at any specified pH and ionic strength, by the relationship:

$$\Delta_r G°'(\mathrm{pH},I) = -RT\ln K' \tag{2}$$

From Eq. (2), the apparent equilibrium constants may be easily calculated from the $\Delta_r G°'$ values, and *vice versa*.

The Gibbs free energies of the activated states, $\Delta G^{\ddagger}$, are connected with kinetic constants, k, by the relationship:

$$\Delta G^{\ddagger} = -RT\ln k \tag{3}$$

By subtracting the sum of the standard transformed Gibbs energies of formation of the reactants, $\Delta_f G°'$, from the sum of the standard transformed Gibbs energies of formation of the products, it is possible to determine the standard transformed reaction Gibbs energy, $\Delta_r G°'$, of any enzymatic reaction for which the free standard transformed Gibbs energy of formation of all reactants and products are known.[2–4]

$$\Delta_r G°'(\mathrm{pH},I) = \Sigma\Delta_f G°'(\mathrm{products}) - \Delta_f G°'(\mathrm{reactants}) \tag{4}$$

The influence of ionic strength is calculated from:[5,6]

$$\mathrm{p}K_1(I) = \mathrm{p}K_1(I=0) + 0.510651\frac{\sqrt{I}}{1+1.6\sqrt{I}}\Sigma\nu_i z_i^2 \tag{5}$$

where K_1 is the dissociation constant of the reactant at a given ionic strength, ν_i is the stoichiometric number of species i. $\Delta_f G_1°'$ is related to the most basic species of the reactant. From $\Delta_f G_1°'(I)$, the value of $\Delta_f G_1°'(I = 0)$ can be calculated and from this further $\Delta_f G_2°'(I = 0)$, from:

$$\Delta_f G_2°'(I=0) = \Delta_f G_1°'(I) - RT\ln 10p\mathrm{K}_1(I=0) \tag{6}$$

Then the standard transformed Gibbs energy of formation of the mixture of both ionic species is calculated at a given pH and ionic strength:[5,6]

$$\Delta_f G^{\circ,} = -RT\ln\left(\exp\left(-\frac{\Delta_f G_1^{\circ,}}{RT}\right) - \exp\left(-\frac{\Delta_f G_2^{\circ,}}{RT}\right)\right) \tag{7}$$

An expanded form of Eq. (7) is:

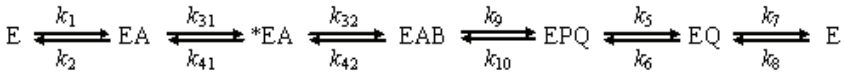
$$\begin{aligned}\Delta_f G^{\circ,} = -2.47897\ln\Big(&\exp\Big(-0.403393\big(\Delta_f G_1'^{\circ}(I=0,\mathrm{pH}\,0) - 2.91482\big(z_1^2 - N_{\mathrm{H}(1)}\big)\frac{\sqrt{I}}{1+1.6\sqrt{I}} + \\ &+ N_{\mathrm{H}(1)}RT\ln 10\mathrm{pH}\big) + \exp\Big(-0.403393\big(\Delta_f G_2'^{\circ}(I=0,\mathrm{pH}\,0) - \\ &-2.91482\big(z_2^2 - N_{\mathrm{H}(2)}\big)\frac{\sqrt{I}}{1+1.6\sqrt{I}} + N_{\mathrm{H}(2)}RT\ln 10\mathrm{pH}\big)\Big)\end{aligned} \tag{8}$$

Most experimental data reported in this work were conducted in aqueous buffers of ionic strength around 0.10 mol dm^{-3}. Since small changes in ionic strength make little contribution to thermodynamic parameters, specification of the ionic strength in the experiments reported was omitted throughout this work.

RESULTS AND DISCUSSION

Binding energies for the coenzymes

Yeast alcohol dehydrogenase, at neutral pH values, operates by an ordered addition of reactants on the aldehyde and a preferred order of addition of reactants on the alcohol side of the reaction, with some dissociation of coenzyme from the central complex EAB.[7] A kinetically detectable isomerization of the enzyme–NAD^+ binary complex occurs on the alcohol side of the reaction (Scheme 1).[1]



Scheme 1.

The standard free energy of binding for NAD^+ to the free enzyme may be calculated from the equilibrium constant k_1/k_2 using Eq. (2). In the same way, the standard free energy of binding for NADH may be calculated from the equilibrium constant k_8/k_7 using the same equation.[7,8] In an Ordered Bi-Bi mechanism, it may be expected that the binding energies for coenzymes to enzyme are independent of the nature of substrates. Table I shows that this indeed is true in most cases. The maximal difference in the binding energies for NAD^+ with widely different substrates is less then 3.7 kJ mol^{-1}, and for NADH less than 2.3 kJ mol^{-1}.

TABLE I. Binding energies for coenzymes operating with different substrates (collected at pH 7.0 and 25 °C, and calculated from the literature[1])

Alcohol	$\Delta G°'$[a] / kJ mol^{-1}	Aldehyde	$\Delta G°'$[b] / kJ mol^{-1}
Ethanol	19.91	Acetaldehyde	28.00
Propan-1-ol	20.72	Butyraldehyde	29.44
Butan-1-ol	21.67	Acetone	27.16
Propan-2-ol	19.54	Butan-2-one	27.51
Butan-2-ol	19.42	DACA[c]	29.23
Allyl alcohol	18.61		
Ethylene glycol	17.91		

[a]Values of $\Delta G°'$ for the binding of NAD^+ calculated from the relationship: $\Delta G°' = -RT \ln (k_1/k_2)$; [b]values of $\Delta G°'$ for the binding of NADH calculated from the relationship: $\Delta G°' = -RT \ln (k_8/k_7)$; [c]dimethylamino-cinnamaldehyde

pH-Dependence of the binding energies for the coenzymes

The binding energies for the coenzymes, NAD^+ and NADH, were calculated for the oxidation of propan-1-ol with the oxidized coenzyme.[9] The binding ener-

gies for NAD^+ were calculated from the equilibrium constant k_1/k_2 (in Scheme 1) with the aid of Eq. (2) (Fig. 1, bottom). The binding energies for NADH were calculated from the equilibrium constant k_8/k_7, again with the aid of Eq. (2) (Fig. 1, top). It is obvious from Fig. 1 that the equilibrium binding energy for NADH changes linearly with pH, with a slope of 2.25 kJ mol^{-1} per pH unit.

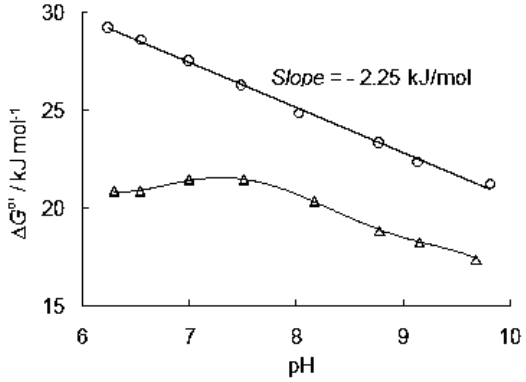


Fig. 1. pH-Dependence of the binding energies for coenzymes. Top: Free energy change for the binding of NADH to the free enzyme. Bottom: Free energy change for the binding of NAD^+ to the free enzyme.

On the other hand, the pH-dependence for the equilibrium binding energy for NAD^+ is a complex function, suggesting that several amino acid side chains on the enzyme participate in the binding of the oxidized coenzyme. In order to analyze this function in detail, the equilibrium constant k_1/k_2 was split into two functions, the second order rate constant k_1, and the first order rate constant k_2. The former was calculated from the initial rate kinetics, from the relationship ln (k_1) = = ln (V_1/K_A), while the latter was calculated from ln (k_2) = ln (k_1) – ln (k_1/k_2).

The pH dependences of the free energy changes associated with both rate constants are shown in Fig. 2. The activation energy, $\Delta G^{\ddagger}$, calculated from k_1

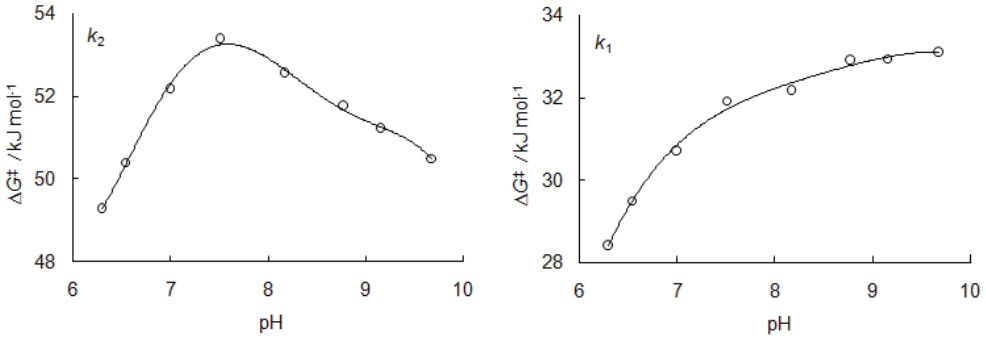


Fig. 2. pH-Dependence of the binding of the oxidized coenzyme. Left: free energy change of activation associated with the rate constant k_2 (s^{-1}) for the dissociation of the enzyme–NAD^+ complex. Right: free energy change of activation associated with the rate constant k_1 (mol^{-1} dm^3 s^{-1}) for the association of NAD^+ with the free enzyme.

(Eq. (3)), follows a complex titration curve governed by the dissociation of at least two amino acid side chains on the protein. The activation energy, $\Delta G^{\ddagger}$, calculated from k_2 (Eq. (3)), also follows a complex titration curve governed by the dissociation of at least two amino acid side chains. Thus, the association of NAD^+ with the free enzyme and dissociation of the enzyme–NAD^+ complex appear to be regulated by the dissociation of 2–4 amino acid side chains on the enzyme.

Temperature dependence of the equilibrium constants for coenzymes

The temperature dependence of the binding energies for the coenzymes was calculated from the initial rate data with the aid of the van't Hoff relationship:

$$\ln K_{eq} = \frac{\Delta S^{\circ}}{R} - \frac{\Delta H^{\circ}}{RT} \quad (9)$$

The K_{eq} values for the k_1/k_2 function were calculated for the oxidation of ethanol and propan-1-ol, while the K_{eq} values for the k_8/k_7 function were calculated only for the reduction of acetaldehyde; both constants may be formally regarded as equilibrium association constants with dimensions mol^{-1} dm^3.

From the straight line in Fig. 3 (bottom), the standard enthalpy, $\Delta H°'$, for the binding of NAD^+ (67.4 kJ mol^{-1}) and the standard entropy, $\Delta S°'$, for the same reaction (158.1 entropy units) can be calculated. Similarly, from the straight line in Fig. 3 (top), the standard enthalpy, $\Delta H°'$, for the binding of NADH (46.1 kJ mol^{-1}), and the standard entropy, $\Delta S°'$, for the same reaction (60.9 entropy units) were calculated.

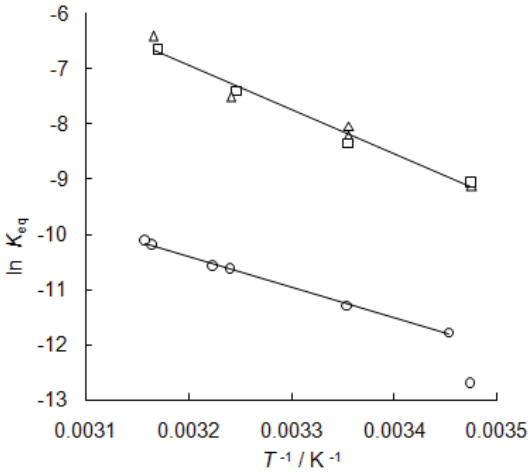


Fig. 3. Temperature dependence of the equilibrium constants for the binding of coenzymes at pH 7.05, calculated from the data of Dickenson and Dickinson.[10,11] Top: binding of NADH to the free enzyme. Bottom: binding of NAD^+ to the free enzyme.

Theoretical data are available in the literature that permit the estimation of the pH-dependence of standard enthalpy of the reaction, $\Delta_r H°'$, and of the standard entropy of the reaction, $\Delta_r S°'$; theoretically, the $\Delta_r H°'$ function is a constant

value for pH 5–9 (45.8 kJ mol^{-1}), while the $\Delta_r S°'$ function increases linearly from 41.1 e.u. at pH 5 to 117.7 e.u. at pH 9.[6] Unfortunately, there are not enough high quality experimental data available for the $\Delta_r H°'$ and $\Delta_r S°'$ values to permit direct comparison between theoretical and experimental results.

Reaction rates in the mechanism of the YADH reaction

The individual rate constants in the overall mechanism of the action of yeast enzyme for the oxidation of ethanol to acetaldehyde were estimated by a combination of the initial rate kinetics and the fitting of the rate constants to the reaction progress curves by appropriate computer fitting programs.[12] The values of these constants and the corresponding changes in the Gibbs free energies are given in Table II.

TABLE II. Thermodynamic properties of yeast alcohol dehydrogenase reaction at pH 7.0 and 25 °C, in the alcohol to aldehyde direction (adapted from the literature[12])

Constant type	Constant	$\Delta G^{\ddagger}$ / kJ mol^{-1}	$\Delta G°'$ / kJ mol^{-1}
Reaction rate	$k_1 = 7\times10^3$ mol^{-1} dm^3 s^{-1}	33.95	–
	$k_2 = 2.1\times10^3$ s^{-1}	54.05	–
Dissociation	$k_2/k_1 = 0.3\times10^3$ mol^{-1} dm^3	–	–20.08
Reaction rate	$k_{31} = 0.39\times10^3$ s^{-1}	58.22	–
	$k_{41} = 2.925\times10^4$ s^{-1}	47.52	–
Dissociation	$k_{41}/k_{31} = 75$	–	10.70
Reaction rate	$k_{32} = 1\times10^3$ mol^{-1} dm^3 s^{-1}	38.77	–
	$k_{42} = 2.11\times10^3$ s^{-1}	54.04	–
Dissociation	$k_{42}/k_{32} = 2.11$ mol^{-1} dm^3	–	–15.26
Reaction rate	$k_9 = 4.0\times10^3$ s^{-1}	52.45	–
	$k_{10} = 3.5\times10^4$ s^{-1}	47.08	–
Dissociation	$k_{10}/k_9 = 8.8$	–	5.37
Reaction rate	$k_5 = 1.09\times10^5$ s^{-1}	49.97	–
	$k_6 = 5\times10^3$ mol^{-1} dm^3 s^{-1}	34.78	–
Dissociation	$k_5/k_6 = 2.2\times10^3$ mol^{-1} dm^3	–	15.18
Reaction rate	$k_7 = 0.39\times10^3$ s^{-1}	58.23	–
	$k_8 = 2.81\times10^4$ mol^{-1} dm^3 s^{-1}	30.51	–
Dissociation	$k_7/k_8 = 14$ mol^{-1} dm^3	–	27.73
			Total: 23.64
	K_{eq}[a] $= 6.8\times10^{-5}$		23.7

[a]Calculated from the steady-state kinetic constants with the aid of the Haldane relationship[7]

From the changes in the standard free energies, a complete energy profile of yeast alcohol dehydrogenase reaction was constructed, which is shown in Fig. 4. There are six forms of enzyme including the free enzyme, with six equilibriums between them. If the complete reaction is followed in the alcohol to aldehyde direction, negative free energy changes are found for the association constant of NAD^+ to free enzyme, E+A $\rightleftarrows$ EA, and for the internal equilibrium *EA +

+ B ⟷ EAB. Positive free energy changes are found for the dissociation constant of the enzyme–NADH complex, EQ ⟷ E+Q, and for the two internal equilibriums, EA ⟷ *EA and EAB ⟷ EPQ. The entire reaction proceeds endothermally in the alcohol to aldehyde direction, with a positive change in the Gibbs free energy of 23.6 kJ mol^{-1}.

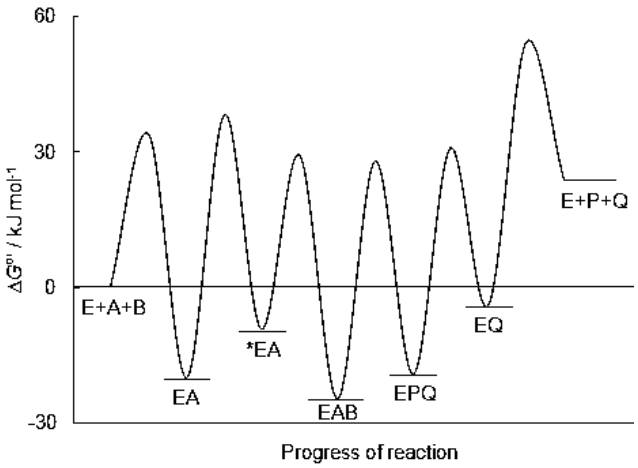


Fig. 4. Thermodynamic energy profile of yeast alcohol dehydrogenase reaction for the oxidation of ethanol to acetaldehyde, at pH 7.0, 25 °C, calculated from the data in Table II.

Binding of oxidized coenzyme to mutant enzymes

The active site of yeast alcohol dehydrogenase is drawn schematically in Fig. 5. The substrate binding pocket is lined with bulky amino acid side chains of Met294, Trp57 and Trp93. A proton relay system is formed by the hydrogen-bonded triad His67···Asp49···Thr84. The pyrophosphate binding region has His47 and Arg369, while the adenine binding pocket is lined with the amino acids Gly224, Phe243, and Ser198.[1]

In the 1990-es, a number of site-directed mutants of yeast alcohol dehydrogenase were constructed, purified, and kinetically characterized in the laboratory of B. V. Plapp.[13–18] The thermodynamic properties of these mutants are summarized in Table III. The standard reaction free energy change for all mutants, $\Delta_r G°'$, differs by less than 10 % from that of the wild type enzyme; this is in accordance with the notion that a catalyst can accelerate a reaction but cannot change its equilibrium. A different situation occurs when the free energy of coenzyme binding is examined. A difference in free energy of binding for both forms of coenzyme between a mutant and a wild type exceeds 50 % when Ser176, Gly183, Asp223, and in the double mutant Asp201 and Gly203 are replaced by another amino acid. This is interesting, since none of these amino acids appears to be directly involved in the binding of coenzymes, except

Asp223; replacement of this amino acid by Gly and the loss of charge, decreases the binding energy by approximately 50 %.

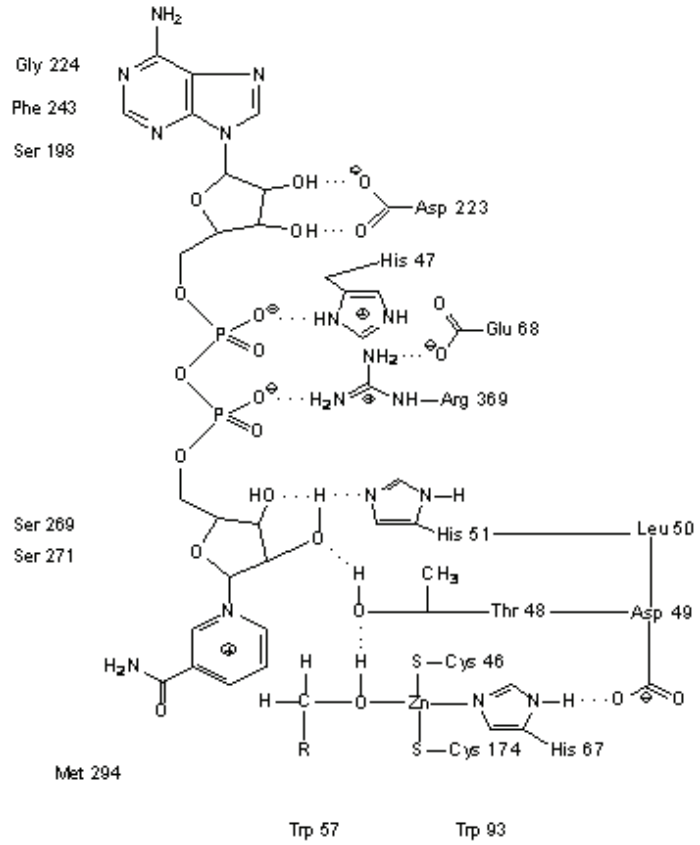


Fig. 5. The active site of yeast alcohol dehydrogenase.
The numeration of the amino acids from the literature.[1]

TABLE III. Thermodynamic properties of mutant enzymes catalyzing the oxidation of ethanol to acetaldehyde, at pH 7.3, 25 °C, calculated from the initial rate kinetic data

No.	Mutant[a]	$\Delta G^{o,b}$ kJ mol^{-1}	$\Delta G^{o,c}$ kJ mol^{-1}	$\Delta_r G^{o,d}$ kJ mol^{-1}	Numeration[e]	Source
1	Wild type	17.34	25.75	20.16	–	13
2	His47Arg	20.47	27.39	18.48	47	14
3	Thr48Ser	16.68	25.31	21.36	48	15
4	Asp49Asn	12.77	20.67	19.96	49	16
5	Trp57Met	14.58	23.99	20.89	57	15
6	Glu68Gln	20.67	25.91	21.68	68	16
7	Trp93Ala	18.70	24.11	—	93	15
8	Ser176Phe	8.78[f]	11.97[f]	18.73	198	17

TABLE III. Continued

No.	Mutant[a]	$\Delta G°'$[b] kJ mol^{-1}	$\Delta G°'$[c] kJ mol^{-1}	$\Delta_r G°'$[d] kJ mol^{-1}	Numeration (as in Fig. 8[e])	Source
9	Gly183Ala	9.55[f]	14.41[f]	18.19	205	17
10	Gly202Ile	13.46	20.29	18.75	224	17
11	Gly203Arg	16.89	27.09	21.28	225	17
12	Asp223Gly	10.255[f]	15.41[f]	19.96	243	18
13	Ser246Ile	15.54	20.67	16.515	269	17
14	Thr48Ser:Trp93Ala	19.96	25.83	18.69	48,93	15
15	Asp201Gly:Gly203Arg	10.255[f]	15.41[f]	17.87	223,225	17

[a]Numeration of amino acids as given by the authors; [b]calculated from the relationship $\Delta G'° = RT\ln(k_1/k_2)$; [c]calculated from the relationship $\Delta G'° = -RT\ln(k_8/k_7)$; [d]calculated from the Haldane relationship; [e]numeration of the amino acids as given in Fig. 5 by Leskovac *et al*;[1] [f]values that are different from the wild type enzyme by more than 50 %

On the other hand, mutation of His47 into Arg does not affect the binding of coenzymes, probably because both amino acids are charged.

CONCLUSIONS

From the evidence summarized in Table I, the ratios of rate constants k_1/k_2 and the rate constants k_8/k_7 may be identified with the equilibrium association constant for the binding of NAD^+ or NADH to the free enzyme, respectively.

From the evidence presented in Figs. 1 and 2, it appears that the association free energy for the binding for NADH to the free enzyme decreases linearly from pH 6–10. On the other hand, the association free energy for the binding for NAD^+ is a complex function, and 2–4 amino acid side chains are involved in the binding of NAD^+. The temperature dependences of the equilibrium constants for the binding of NAD^+ or NADH to the free enzyme are shown in Fig. 3. From the appropriate plots, the enthalpy and entropy for both reactions were calculated.

The energy profile of the entire yeast alcohol dehydrogenase reaction when ethanol is oxidized to acetaldehyde, constructed from the detailed thermodynamic data in Table II, is shown in Fig. 4.

Finally, Table III shows the association free energy for the binding of NAD^+ or NADH to the free enzyme, for 15 single or double mutants of the yeast enzyme, revealing the amino acid side chains important for the binding of the coenzyme.

In conclusion, this communication presents a complete and comprehensive survey of binding data for the enzyme, based only on thermodynamic data. This is the first survey of this kind reported so far in the literature.

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ИЗВОД

ВЕЗИВАЊЕ КОЕНЗИМА НА АЛКОХОЛ-ДЕХИДРОГЕНАЗУ ИЗ КВАСЦА

ВЛАДИМИР ЛЕСКОВАЦ[1], СВЕТЛАНА ТРИВИЋ[2], ДРАГИЊА ПЕРИЧИН[1], МИРА ПОПОВИЋ[2] и ЈУЛИЈАН КАНДРАЧ[3]

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У овом раду је истраживано везивање коензима на алкохол-дехидрогеназу из квасца (EC 1.1.1.1). Главни критеријуми су били промене у стандардним слободним енергијама за поједине реакционе ступњеве, унутрашње константе равнотеже и промена слободне енергије укупне реакције. Израчунавања су спроведена за нативну врсту ензима при pH 6–9, и за 15 врста различитих мутантних ензима, са једном или две мутације, при pH 7,3. Обиље теоријских и експерименталних података омогућило је да се детаљно истражи везивање коензима на ензим.

(Примљено 26. маја, ревидирано 23. јула 2009)

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