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INFLUENCE OF TRIS(HYDROXYMETHYL)AMINOMETHANE ON KINETIC MECHANISM OF YEAST ALCOHOL DEHYDROGENASE

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Acetaldehyde, propionaldehyde, glyceraldehyde-3-*P* and 4-dimethylaminocinnamaldehyde form Schiff bases in Tris · HCl buffers; the rates of formation and dissociation of Schiff bases, and equilibrium constants for their formation are very similar for the first three aldehydes. The steady-state kinetic constants for the yeast alcohol dehydrogenase-catalyzed reaction, propan-1-ol + $NAD^+ \rightleftharpoons$ propionaldehyde + NADH + H^+, have been determined in several Tris · HCl buffers of increasing concentration at pH 8.1. In the forward direction, oxidation of alcohol, most kinetic constants are increased by increasing concentrations of Tris. In the reverse direction, reduction of aldehyde, substrate, NADH, Tris and Schiff base were equilibrated before enzyme reaction was started. It was found that Schiff base, rather than Tris, binds to free enzyme competitively with respect to NADH. Tris and Schiff base do not influence the binding of aldehyde to enzyme in any way.

Keywords: Yeast alcohol dehydrogenase; Schiff base

Abbreviations: YADH – yeast alcohol dehydrogenase; DACA – 4-dimethyl-amino-cinnamaldehyde; AA – acetamide.

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INTRODUCTION

Tris(hydroxymethyl)aminomethane is often used as a buffer for the isolation, purification and the study of steady-state kinetics of NAD^+-dependent alcohol dehydrogenases; carbonyl compounds are common substrates of this group of enzymes. Carbonyl compounds readily react with Tris buffer to form Schiff bases; early reports in biochemical literature on this reaction include the papers of Duggan *et al.*,[1] Dickinson and Dalziel[2] and Ogilvie and Whitaker.[3] Schiff base in Tris readily polymerises in alkaline medium.[4] These reactions of Tris base with carbonyl substrates have been often neglected in steady-state kinetic studies of alcohol dehydrogenases, which has led to misinterpretation of kinetic data in numerous cases.[5–9]

Therefore, we have performed a systematic study of the influence of Tris buffer on the steady-state kinetic parameters of yeast alcohol dehydrogenase (EC 1.1.1.1, constitutive, cytoplasmic), isolated from *Saccharomyces cerevisiae*. The yeast enzyme served as a model for other alcohol dehydrogenases.

MATERIALS AND METHODS

Materials

Yeast alcohol dehydrogenase (lyophilized powder) was obtained from Boehringer (Mannheim, Germany). Specific activity of enzyme with ethanol was 300 U/mg of enzyme protein, estimated at pH 9, according to Bergmeyer.[10] The concentration of enzyme protein in solution was determined according to Hayes and Velick,[11] and the concentration of enzyme active sites by the fluorescent method of Leskovac *et al.*[12] NAD^+ and NADH were purchased from Sigma (St. Louis, USA), alcohol and aldehyde substrates were purchased from Aldrich (Steinheim, Germany) and distilled before use. Tris · HCl (99.9+%) and DACA (98%) were obtained from Aldrich (Steinheim, Germany) and used without further purification. All other chemicals were of the highest grade purity, obtained from commercial sources.

Methods

Absorption spectra were recorded from 230–800 nm in a spectrophotometer SPECORD UV VIS, Carl Zeiss (Jena, Germany), in themostated cuvette holders at 25°C. Concentrations of substrates were determined from their

molar extinction coefficients at pH 7.0 ($M^{-1}\,cm^{-1}$): NAD^+ 18000 at 260 nm, NADH 6200 at 340 nm[10] and DACA 31000 at 400 nm;[13] the concentration of aldehyde and alcohol substrates was determined enzymatically.[10]

Enzyme reaction rates in the forward direction, oxidation of alcohols, were determined from the initial linear portion of reaction progress curves in sodium phosphate buffer pH 6.0–8.0, sodium pyrophosphate buffer pH 8.0–9.1 or Tris · HCl buffer pH 7.0–9.0, supplemented with 0.5 mM EDTA. The following equation was fitted to initial rate data with the SEQUEN Fortran program of Cleland:[14]

$$v_0/e_0 = \frac{V_1[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]}, \quad (1)$$

where v_0 is the initial rate ($M\,s^{-1}$), e_0 the concentration of enzyme active sites (M), V_1 the catalytic constant (s^{-1}), K_a and K_b the Michealis constants for NAD^+ and alcohols (M), K_{ia} the inhibitory constant for NAD^+ (M), and [A] and [B] the concentrations of NAD^+ and alcohols, according to the nomenclature of Cleland.[15] In the reverse direction, reduction of aldehydes, kinetic constants were calculated in an analogous fashion; in this direction, V_2 was the catalytic constant (s^{-1}), K_q and K_p Michaelis constants for NADH and aldehydes (M), K_{iq} the inhibitory constant for NADH (M), and [Q] and [P] the concentrations of NADH and aldehydes (M), respectively.

Dissociation constants of enzyme · NADH complexes and dissociation constants of NADH from enzyme · NADH · acetamide complexes in various buffers (Table V) were determined as previously described.[16,17]

RESULTS AND DISCUSSION

Formation of Schiff Base

Acetaldehyde and Propionaldehyde

Acetaldehyde and propionaldehyde have a single absorption band at 280 nm ($\varepsilon = 9.5$ and $9.6\,M^{-1}\,cm^{-1}$, respectively) in aqueous buffers; this peak gradually decreases in Tris · HCl buffer, which was interpreted as formation of Schiff base between Tris and aldehyde:[3,4]

$$RCHO + R'_3CNH_2 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} RCH{=}NCR'_3 + H_2O \quad (2)$$

Since the peak at 280 nm disappears completely in the presence of large excess of Tris, it was assumed that the Schiff base does not absorb at 280 nm. Figure 1 shows kinetics of Schiff base formation between acetaldehyde or propionaldehyde and Tris.

Under the pseudo-first order conditions, with large excess of Tris over aldehyde (data not shown), and under the second-order conditions with equal starting concentrations of Tris and aldehyde (Figure 1A), reaction appears kinetically homogeneous. A strict adherence to the second-order rate law (Figure 1A) confirms the stoichiometry of equation (2) and indicates that carbinolamine intermediate is kinetically insignificant.[3,4]

Equilibrium constants (K_{eq}) for reaction (2) were determined at pH 8.0–8.1, from the relationship:

$$\text{Log}([P_0]/[P_{eq}] - 1) = \text{Log } K_{eq} + \text{Log}[\text{Tris-base}_{eq}], \qquad (3)$$

where $[P_0]$ and $[P_{eq}]$ are starting and equilibrium concentrations of aldehyde, and $[\text{Tris-base}_{eq}]$ the concentration of Tris base at equilibrium. It was assumed that absorbancy at 280 nm is a measure of the concentration of free aldehyde (Figure 1B). Bimolecular rate constants for the formation of Schiff base (k_f) were determined under pseudo-first order conditions, with large excess of Tris over aldehyde, at pH 8.1. Reaction progress curves for

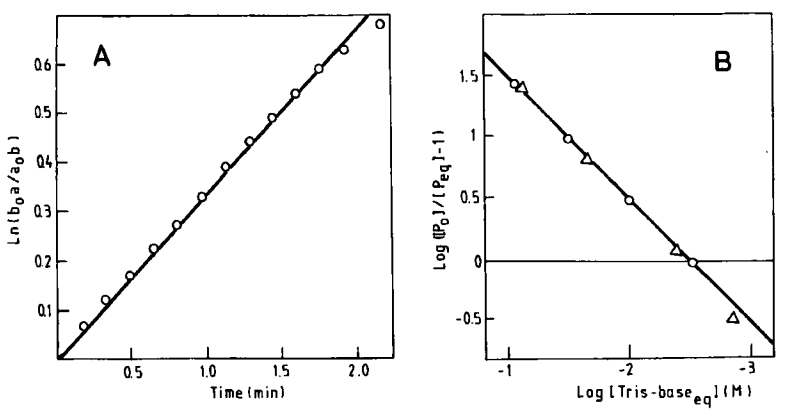

FIGURE 1 Kinetics of Schiff base formation between acetaldehyde or propionaldehyde and Tris. (A) Propionaldehyde (20 mM) was dissolved in 26 mM Tris · HCl and 0.1 M sodium pyrophosphate buffer, pH 8.1; the concentration of reactants was fitted to eqn: $\ln(b_0a/a_0b) = k_f(a_0-b_0)t$, where a_0 and b_0 are starting concentrations of aldehyde and Tris base, and a and b their concentrations after t minutes. (B) Acetaldehyde (21 mM, △) or propionaldehyde (14.6 mM, ○) were mixed with increasing concentrations of Tris · HCl buffer (10–200 mM), pH 8.1, and extinction at 280 nm and pH recorded after the equilibrium was attained; K_{eq} was calculated from equation (3).

the disappearance of aldehyde band in Tris at 280 nm were recorded and k_f determined from the same, with the aid of Guggenheim's method[18] (data not shown). Monomolecular rate constants for the reverse reaction (k_r) were calculated from the relationship: $K_{eq} = k_r/k_f$. Table I summarizes K_{eq}, k_f and k_r values for acetaldehyde, propionaldehyde and DACA; for comparison, similar values for glyceraldehyde-3-*P* are included from the literature.[3]

At pH 8.0, the propionaldehyde peak at 280 nm decreases rapidly in the presence of excess Tris, and a novel peak at 240 nm develops very slowly (Figure 2A). At pH 11.0, the peak at 240 nm develops instantaneously, with an apparent absorption coefficient of 230 optical units/M of aldehyde (data not shown). The latter spectral change was interpreted as formation of various polymerization products;[4] this polymerization is very slow at pH 7.0–8.5 and does not influence the determination of kinetic data presented in Table I.

DACA

DACA has a strong absorption band at 400 nm ($\varepsilon = 31000\,M^{-1}\,cm^{-1}$).[13] DACA (41 μM) reacts with 0.375 M Tris · HCl buffer pH 8.1, which is evident from the gradual increase of its absorbancy at 470 nm and decrease at 400 nm. Since the spectral changes were characterized by two isosbestic points, it was concluded that a single product was formed, a Schiff base between DACA and Tris (Figure 2B). With the aid of Guggenheim's method,[18] it was found that changes in absorbancy at 470 nm obey the pseudo-first order kinetic law with a rate constant of $1.0\,M^{-1}\,min^{-1}$ at pH 8.1, which was ascribed to the bimolecular rate constant for the formation of Schiff base (k_f) (Table I). Since the spectrum of DACA at 400 nm did not disappear in the presence of excess Tris, we were unable to determine K_{eq} for this reaction.

Geminal Diols

The carbonyl group of aliphatic aldehydes is readily hydrated to geminal diols in aqueous buffers, whereby free aldehydes are true substrates of dehydrogenases, while geminal diols are not.[20,21] Table I shows the K_{eq}, k_f and k_r values for acetaldehyde, propionaldehyde and glyceraldehyde-3-*P*, calculated by correcting the concentration of free aldehydes for hydration; corrected K_{eq} and k_f values are much higher for glyceraldehyde-3-*P*, because this aldehyde is 97% hydrated,[3] while acetaldehyde and propionaldehyde are only 60–70% hydrated at pH 7–8.[21] As might be expected, k_r values are unaffected by hydration (Table I).

TABLE I Equilibrium and rate constants for the reaction of aldehydes with Tris base at 25°C

Aldehyde	pH	*Uncorrected for geminal diol*			*Corrected for geminal diol*		
		K_{eq} (M^{-1})	k_f ($M^{-1}\,min^{-1}$)	k_r (min^{-1})	K_{eq} (M^{-1})	k_f ($M^{-1}\,min^{-1}$)	k_r (min^{-1})
Acetaldehyde	8.0	343 ± 20	20.2 ± 1.8	0.059	629	37	0.059
Propionaldehyde	8.1	306 ± 18	15.3 ± 3.5	0.05	497	25	0.050
DACA	8.10	—	1.0	—	—	—	—
Glyceraldehyde-3-*P*[a]	7.45	369 ± 12	18.2	0.056	11000	546	0.050
Glyceraldehyde-3-*P*[a]	8.50	369 ± 12	5.5	0.014	11000	165	0.016

[a] Data taken from Ogilvie and Whitaker.[3]

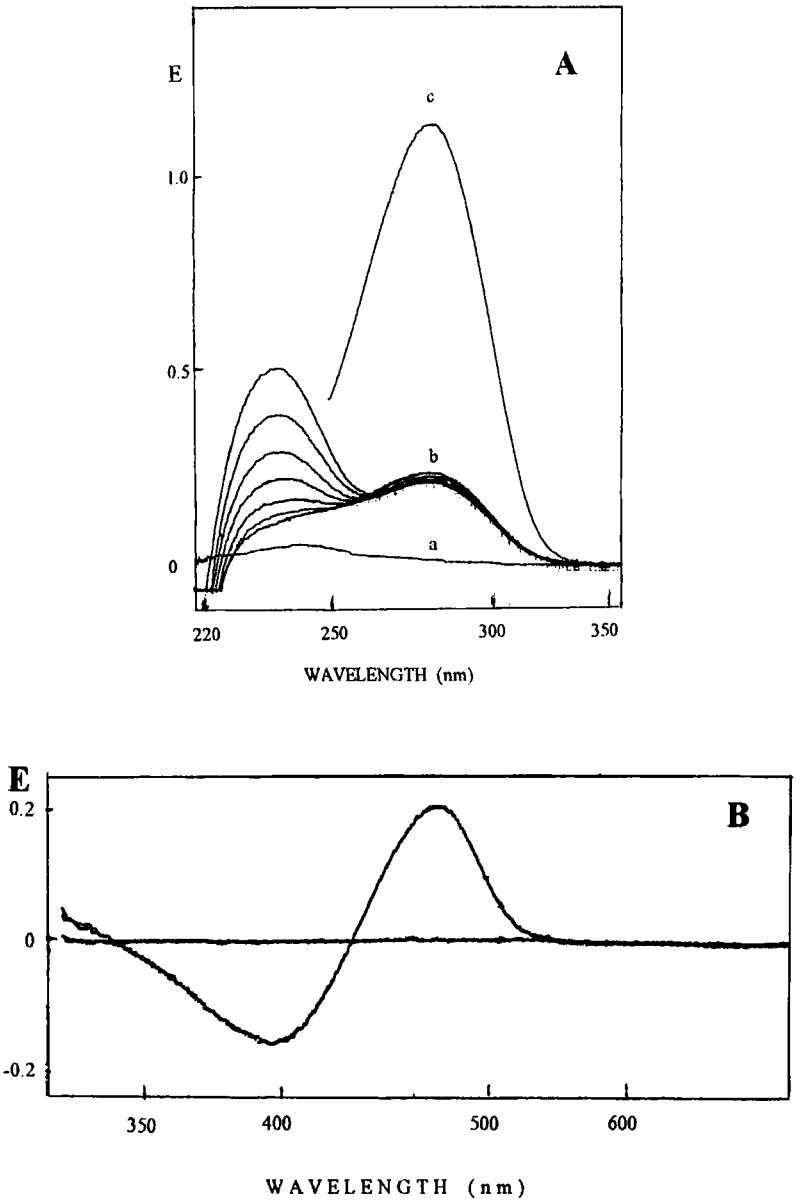

FIGURE 2 (A) Spectral changes of propionaldehyde in Tris · HCl buffer. (a) Base line. (b) Propionaldehyde (0.2 M) was mixed with 0.5 M Tris · HCl buffer, pH 8.1, and the spectrum recorded after 1, 6, 15, 30, 45, 62 and 80 mins. (c) Spectrum of propionaldehyde (0.1 M) in 0.1 M Na-pyrophosphate buffer, pH 8.1. (B) Difference spectrum between DACA (41 μM) in 0.1 M sodium phosphate buffer, pH 8.1 (reference cuvette) and DACA (41 μM) in 0.375 M Tris · HCl buffer, pH 8.1 (sample cuvette), recorded 20 min after mixing DACA with Tris; light path 1 cm.

Steady-State Kinetics of YADH in Tris · HCl Buffers

Oxidation of Propan-1-ol

The steady-state kinetic constants for the YADH-catalyzed reaction, propan-1-ol + $NAD^+ \rightarrow$ propionaldehyde + NADH + H^+, were determined in Tris · HCl buffers of increasing ionic strength, from 0 to 5 M (0 to 0.24 μ) at pH 8.1 (Table II). In the forward direction, oxidation of alcohol, K_b and V_1/K_a constants are practically unaffected by Tris buffer, while V_1, K_a, K_{ia}, V_1/K_b and V_1K_{ia}/K_a constants are increased 3–6-fold in going from zero to 0.3 M Tris · HCl (0.145 μ). Kinetic mechanism of yeast alcohol dehydrogenase in the forward direction, oxidation of propan-1-ol, is steady-state random or preferred ordered;[19] in this mechanism, monomolecular (V_1, V_1K_{ia}/K_a) and bimolecular (V_1/K_a, V_1/K_b) kinetic constants cannot be ascribed to individual rate constants.[22] Therefore, we were unable to assess the influence of increasing concentrations of Tris on individual rate constants. Yet, it appears that Tris buffer affects to some extent most rate constants in the forward direction, including the binding of NAD^+.

Reduction of Propionaldehyde

Steady-state kinetic constants for the reverse reaction, propionaldehyde + NADH + $H^+ \rightarrow$ propan-1-ol + NAD^+, were determined in a similar fashion in Tris · HCl buffers of increasing ionic strength, at pH 8.1 (Table III). Special care was taken to obtain kinetic constants in Tris buffers, as propionaldehyde readily forms a Schiff base with Tris. Kinetic constants were determined in 0.03 M and 0.1 M Tris · HCl, by varying the concentration levels of both substrates, propionaldehyde and NADH. The application of concentrated Tris buffers (>0.1 M) reduced the concentration of free aldehyde well below its K_m and, therefore, afforded inaccurate initial reaction rates. Initial reaction rates were measured after the aldehyde was preincubated in Tris, in order to allow for equilibration between free aldehyde and Schiff base. The actual concentrations of free aldehyde and Schiff base in the test were calculated by the application of K_{eq} value from Table I. Thus, the concentrations of reactants in tests varied from: free aldehyde 0.05–0.62 mM, Schiff base 0.7–3.7 mM, and NADH 16–100 μM (Table III). In Table III, the steady-state kinetic constants obtained[19] in 0.1 M sodium phosphate buffer pH 8.12 are compared with kinetic constants obtained, as described above, in Tris · HCl buffers pH 8.07. It is clear that most constants are very similar in both buffers, with the exception of K_q, V_2/K_q and V_2K_{iq}/K_q constants. Kinetic mechanism of YADH in the

TABLE II Influence of Tris · HCl buffer on steady-state kinetic constants of YADH-catalyzed reaction: propan-1-ol + NAD^+ → propionaldehyde + NADH + H^+

Buffer	μ	0[a]	0.0163	0.051	0.098	0.145	0.237
pH		8.1[a]	8.13	8.10	8.10	8.13	8.12
V_1	s^{-1}	67	88 ± 8	119 ± 10	167 ± 15	189 ± 17	154 ± 8
K_a	mM	0.34	0.48 ± 0.09	0.61 ± 0.12	1.00 ± 0.20	1.18 ± 0.20	0.68 ± 0.09
K_{ia}	mM	0.35	0.49 ± 0.07	0.77 ± 0.13	1.31 ± 0.20	1.69 ± 0.28	1.84 ± 0.33
K_b	mM	59.5	59.1 ± 9.5	43.5 ± 7.3	41.4 ± 7.0	39.5 ± 7.4	23.5 ± 3.7
V_1/K_a	$mM^{-1}s^{-1}$	176	181 ± 19	195 ± 22	187 ± 20	160 ± 14	225 ± 20
V_1/K_b	$mM^{-1}s^{-1}$	1.13	1.48 ± 0.10	2.73 ± 0.23	4.20 ± 0.30	4.77 ± 0.49	6.53 ± 0.73
V_1K_{ia}/K_a	s^{-1}	70	89	151	219	270	414

[a] Calculated by extrapolation to zero Tris buffer.

reverse direction, reduction of propionaldehyde, is strictly ordered,[19] in this mechanism, V_2/K_q is equal to the bimolecular rate constant for the association of NADH with free enzyme and V_2K_{iq}/K_q is equal to the monomolecular rate constant for the dissociation of the E · NADH complex.[22] Tris · HCl buffer decreases both rate constants by 3-fold, thus leaving the dissociation constant $K_{E,NADH}$ (K_{iq}) unaffected (Table III). Therefore, it appears that Tris · HCl binds to free enzyme, competitively with respect to NADH. It should be noted that kinetic constants in Table III are obtained in the presence of Tris · HCl and Schiff base of varying concentrations, thus raising the question as to which of the two compounds binds competitively with respect to NADH. Since the dissociation constant $K_{E \cdot AA,NADH}$ (obtained in the absence of Schiff base) is unaffected by Tris (Table IV), it is concluded that Schiff base, rather than Tris, binds to the enzyme competitively with respect to NADH. Also, it appears that Tris · HCl or Schiff base do not influence the binding of aldehyde to enzyme in any way.

TABLE III Steady-state kinetic constants for YADH-catalyzed reaction: propionaldehyde + NADH + H^+ → propan-1-ol + NAD^+, in 0.1 M sodium phosphate and Tris · HCl buffer

Buffer		*Phosphate*[a]	*Tris*[b]
pH		8.12	8.07
V_2	s^{-1}	930	962 ± 179
K_q	μM	45.4	156 ± 40
K_{iq}	μM	43.5	48.8 ± 6.4
K_p	mM	1.53	1.08 ± 0.26
V_2/K_q	$\mu M^{-1} s^{-1}$	20.5	6.15 ± 0.47
V_2/K_p	$\mu M^{-1} s^{-1}$	608	891 ± 52
V_2K_{iq}/K_q	s^{-1}	891	299

[a] Data taken from Leskovac *et al.*[19]

[b] Kinetic constants determined statistically from 24 initial rate data measured in 0.03 and 0.1 M Tris · HCl buffer. Concentration of reactants in 0.03 M Tris (mM); free aldehyde (Schiff base): 0.13 (0.7), 0.19 (1.0), 0.32 (1.7) and 0.63 (3.3), in the presence of NADH (μM); 15.6, 22.2, 33.1 or 83. Concentration of reactants in 0.1 M Tris (mM); free aldehyde (Schiff base): 0.05 (0.76), 0.07 (1.14), 0.12 (1.9) and 0.25 (3.67), in the presence of NADH (μM): 40 or 100.

TABLE IV Dissociation constants of various complexes of yeast alcohol dehydrogenase with NAD^+ or NADH at pH 8.1

Buffer (M)	K_{E,NAD^+} (μM)	$K_{E,AA,NADH}$ (μM)
Tris · HCl (0.10)	—	5.17 ± 0.23
Tris · HCl (0.50)	—	6.83 ± 0.38
Tris · HCl (0.053)	252 ± 18	—
Tris · HCl (0.492)	504 ± 87	—
Na-pyrophosphate (0.05)	302 ± 30	5.28 ± 0.45

Dissociation Constants of YADH,NAD^+ and YADH · AA,NADH Complexes

Table IV shows the values of the dissociation constants K_{E,NAD^+} and $K_{E \cdot AA,NADH}$, obtained at pH 8.1, in 0.05 M sodium pyrophosphate buffer, and in 0.05, 0.10, or 0.5 M Tris · HCl buffer.

Steady-State Kinetics of YADH in Potassium Phosphate Buffers

Table V shows the steady-state kinetic constants for the YADH-catalyzed reaction, propan-1-ol + $NAD^+ \rightarrow$ propionaldehyde + NADH + H^+, determined in 0.1 and 1.0 M potassium phosphate buffer, pH 7.2. In the forward direction, oxidation of alcohol, increasing concentration of phosphate buffer increases V_1, K_a, K_{ia}, V_1/K_b and V_1K_{ia}/K_a constants by 2–3-fold, leaving other constants unaffected. Thus, it appears that the influence of increasing concentrations of orthophosphate and Tris · HCl buffer on the forward reaction are very similar (Tables II and V). In the reverse direction, reduction of aldehyde, increasing concentration of phosphate buffer increases K_{iq} and V_2K_{iq}/K_q constants by 2–3-fold, leaving other constants unaffected. Thus, it appears that phosphate buffer increases only the rate of dissociation of E · NADH complex (see above).

In conclusion, it is interesting to note that Tris · HCl and phosphate buffers have a very similar effect on the kinetic mechanism of yeast enzyme in

TABLE V Influence of potassium phosphate buffer, pH 7.2, on steady-state kinetic constants of YADH-catalyzed reaction: propan-1-ol + $NAD^+ \rightleftharpoons$ propionaldehyde + NADH + H^+

Buffer		0.1 M	1.0 M
V_1	s^{-1}	25.1 ± 3.6	65.7 ± 8.3
K_a	μM	276 ± 71	614 ± 138
K_{ia}	μM	216 ± 73	584 ± 211
K_b	mM	44.2 ± 13.5	31.5 ± 9.8
V_1/K_a	$mM^{-1}s^{-1}$	90.8 ± 10.9	106.9 ± 11.1
V_1/K_b	$mM^{-1}s^{-1}$	0.57 ± 0.10	2.11 ± 0.42
V_1K_{ia}/K_a	s^{-1}	19.6	62.5
V_2	s^{-1}	987 ± 121	980 ± 85
K_q	μM	85.8 ± 15.7	99.7 ± 14.1
K_{iq}	μM	12.9 ± 3.0	34.8 ± 7.7
K_p	mM	3.0 ± 0.5	1.2 ± 0.2
V_2/K_q	$\mu M^{-1}s^{-1}$	11.5 ± 0.8	9.8 ± 0.6
V_2/K_p	$mM^{-1}s^{-1}$	331 ± 18	805 ± 71
V_2K_{iq}/K_q	s^{-1}	148	342
K_{eq} ($\times 10^3$)[a]		0.103	0.152

[a] $K_{eq} = V_1K_{iq}K_p/V_2K_{ia}K_b$.

the forward direction, which we ascribe to nonspecific salt effects. On the other hand, in the reverse direction, Schiff base decreases the rates of formation and dissociation of the E · NADH complex, while phosphate buffer increases only the rate of the former reaction.

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