Potassium Is an Activator of Homoisocitrate Dehydrogenase from *Saccharomyces cerevisiae*[†]

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ABSTRACT: Potassium is an activator of the reaction catalyzed by homoisocitrate (HIc) dehydrogenase (HIcDH) from *Saccharomyces cerevisiae* with either the natural substrate, homoisocitrate, or the slow substrate isocitrate. On the basis of initial velocity studies, the selectivity of the activator site for monovalent ions was determined. Potassium is the best activator, and NH_4^+ and Rb^+ are also activators of the reaction, while Cs^+, Li^+, and Na^+ are not. Chloride inhibits the reaction, while acetate is much less effective. Substitution of potassium acetate for KCl changes the kinetic mechanism of HIcDH from a steady state random to a fully ordered mechanism with the binding of MgHIc followed by K^+ and NAD. The change in mechanism likely reflects an apparent increase in the affinity of enzyme for MgHIc as a result of elimination of the inhibitory effect of Cl^-. The V/K_{NAD} pH−rate profile in the absence of K^+ exhibits a >10-fold decrease in the affinity of enzyme for NAD upon deprotonation of an enzyme side chain with a pK_a of about 5.5−6. On the other hand, the affinity for NAD is relatively constant at high pH in the presence of 200 mM KCl. Since the affinity of the dinucleotide decreases as the enzyme group is protonated and the effect is overcome by a monovalent cation, the enzyme residue may be a neutral acid, aspartate or glutamate. Data suggest that K^+ replaces the proton, and likely binds to the enzyme residue, the pyrophosphoryl moiety of NAD, or both. Viscosity and solvent deuterium isotope effects studies suggest the isomerization of E−MgHIc binary complex limits the rate in the absence of K^+.

Homoisocitrate dehydrogenase (3-carboxy-2-hydroxyadipate dehydrogenase, EC 1.1.1.87) (HIcDH[1]) catalyzes the fourth reaction of the α-aminoadipate pathway (AAA) for lysine biosynthesis, the NAD-dependent conversion of homoisocitrate (HIcDH) to α-ketoadipate (α-Ka) (*1*). The dehydrogenase is a member of the family of pyridine nucleotide-dependent β-hydroxyacid oxidative decarboxylases, and is a metal ion-dependent enzyme that selectively binds the MgHIc chelate complex (*2*). The kinetic mechanism of HIcDH has been determined recently (*2*), and is a steady-state mechanism with random addition of MgHIc and NAD, but with a preferred ordered release of CO_2, α-Ka, and NADH. Given that the HIcDH reaction is unique to lysine synthesis in fungi, it is a potential target for the development of lead compounds for new antifungal drugs.

During the course of routine assays, it was found by the authors that HIcDH is activated by K^+. This is also the case for tartrate dehydrogenase (TDH) (*3*), another member of the family, which has 36% sequence identity to HIcDH. TDH has a requirement for a monovalent metal ion for activation, and a divalent metal ion for binding of D-malate prior to its oxidative decarboxylation. Dipolar coupling between $^{113}Cs^+$ and Mn^{2+} in ESEEM studies of TDH suggested the monovalent metal ion is located in the active site and may be coordinated to the substrate (*3*). However, the mechanism of K^+ activation of HIcDH is still unknown. It is important to determine whether the activation by K^+ is structural (nonactive site) or important for binding and/or catalysis of the HIcDH. If it is important for binding and/or catalysis, this must be taken into account in the development of good inhibitors of the enzyme.

A large group of enzymes requiring a monovalent cation (M^+) for optimal activity have been discovered in the plant and animal world (*4, 5*). K^+ or Na^+ is the preferred M^+ in enzymes since enzymes take advantage of the ready availability of Na^+ outside the cell and K^+ inside the cell to optimize their catalytic function. The mechanism of M^+ activation can be determined from kinetic investigation and structural studies. One possibility is that the M^+ anchors the substrate to the active site of the enzyme, often acting in tandem with a divalent cation like Mg^{2+}. In this case, the M^+ is usually essential for catalysis. The second possibility is that the M^+ enhances enzyme activity through conforma-

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[1] Abbreviations: HIcDH, homoisocitrate dehydrogenase; AAA, α-aminoadipate pathway; α-Ka, α-ketoadipate; NAD, nicotinamide adenine dinucleotide (the (+) charge is omitted for convenience); NADH, reduced nicotinamide adenine dinucleotide; NADD, reduced nicotinamide adenine dinucleotide with deuterium in the 4-*R* position; TDH, tartrate dehydrogenase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl) imino-tris(hydroxymethyl) methane; Tris, tris(hydroxymethyl) aminomethane; Ic, isocitrate; KOAc, potassium acetate; TMAC, tetramethylammonium chloride; CPM, 3-carboxypropylidenemalate; MgHIc, chelate complex of magnesium and homoisocitrate; D_2O, deuterium oxide; DCl, deuterium chloride; NaOD, sodium deuteroxide; SKIE, solvent kinetic isotope effect.

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tional transitions triggered upon binding to a site where the M^+ makes no direct contact with substrate. In the latter case, the M^+ is not absolutely required for catalysis (*6*).

In this paper, we use initial velocity studies, pH−rate profiles, and isotope effects to probe the mechanism of activation by K^+ of HIcDH. Data suggest that the major effect of K^+ is to increase the affinity of enzyme for NAD at high pH.

MATERIALS AND METHODS

Chemicals. Potassium acetate (KOAc), KCl, LiCl, RbCl, CsCl, and tetramethylammonium hydroxide were obtained from Sigma. NaCl and $MgCl_2$ were purchased from EMD, and NH_4Cl was from Fisher. β-NAD and β-NADH were from USB. Hepes, Tris, Bis-Tris, and Ches buffers were from Research Organics, Inc. Deuterium oxide (D_2O) (99 atom % D) was purchased from Cambridge Isotope Labotories, Inc. α-Ketoadipate, HIc and A-side NADD were prepared according to published procedures (*7*).

Enzyme Assay. The activity of HIcDH was measured using a Beckman DU 640 spectrophotometer to monitor the increase or decrease in absorbance at 340 nm as NAD is reduced or NADH is oxidized. All assays were carried out at 25 °C. A unit of enzyme activity is defined as the amount of enzyme catalyzing the production or utilization of 1 μmol of NADH per min at 25 °C. Typical assays contained in a 1 mL volume: 100 mM Tris or Hepes, pH 8.0, and variable concentrations of MgHIc and NAD in the presence or absence of potassium ion. At pH 8, the final enzyme concentration in the presence of K^+ was 1.5 μg/mL, while it was 90 μg/mL in the absence of K^+.

Initial Velocity Studies. Reaction rates were measured at fixed concentrations of MgHIc (4.2 μM) and NAD (0.5 mM) with 200 mM chloride salts, or in the absence of chloride. Li^+, Na^+, K^+, NH_4^+, Rb^+, Cs^+, and Cl^- were used as additives to test their effects on HIcDH activity. The initial rates were also measured at fixed concentrations of MgHIc (4.2 μM) and NAD (0.5 mM) with different concentrations of KCl or KOAc to obtain the activation constant for K^+ and the inhibition constant for Cl^- or Ac^-.

The initial rate was measured as a function of MgHIc (2.1, 4.2 7.5, and 21 μM) at different fixed levels of NAD (8, 16, 22, and 35 mM) in the absence of KCl. In the presence of KCl, the initial rate was also measured as a function of MgHIc as above at different fixed levels of NAD (0.2, 0.3, 0.5, and 2 mM) and a fixed concentration of KCl (5 mM). The experiment was then repeated at several additional KCl concentrations (15, 150, and 350 mM). The obtained kinetic parameters V, K_{iMgHIc}, V/K_{MgHIc}, and V/K_{NAD} were then plotted against the concentration of KCl.

The initial velocity patterns with KOAc as an activator were obtained by measuring the initial rate as a function of MgHIc (1.2, 2.1, 4.2, and 7.5 μM) at different fixed levels of NAD (0.5, 1, 2, and 5 mM) and a fixed concentration of KOAc (5 mM). The experiment was then repeated at several additional KOAc concentrations (10, 20, and 30 mM).

Product and Dead-End Inhibition Studies in the Absence of K^+. Initial velocity patterns were obtained by measuring the initial rate at different concentrations of one reactant, with the concentration of the other reactants fixed at either K_m or $10K_m$, and at different fixed concentrations of the inhibitor, including zero. In the case of measuring the product inhibition by NADH, because of the high absorbance at 340 nm, data were collected at 363 nm using a ε_{363} of 3,110 M^{-1} cm^{-1}. An estimate of the K_{iNADH} for the E−MgHIc−NADH ternary complex was obtained using saturating MgHIc, fixing NAD at 1 mM ($0.1K_m$), and varying the inhibitor concentration. The appK_i was estimated by Dixon analysis, a plot of $1/v$ versus **I** was extrapolated to $1/v$ equal to zero, and then the value of **I** was divided by 1.1.

Solvent Deuterium and Viscosity Effects in the Absence of K^+. Initial velocities were measured in H_2O and D_2O. For rates measured in D_2O, substrates and buffers were first dissolved in a small amount of D_2O and lyophilized overnight to remove exchangeable protons. The lyophilized powders were then dissolved in D_2O to give the desired concentration, and the pD was adjusted using NaOD. A value of 0.4 was added to the pH meter reading to calculate pD (*8*). Data were obtained by varying one substrate at a fixed concentration ($10K_m$) of the other one. The isotope effects were obtained by direct comparison of initial rates in H_2O and D_2O at pH(D) 8.0, which is in the pH-independent range of the V and V/K pH−rate profiles. Reactions were initiated by adding 5 μL of enzyme solution (0.9 mg/mL) in H_2O, such that the final percentage of D_2O was about 99%.

Initial velocities were determined in H_2O at a relative viscosity of 1.24 at pH 8.0 and 25 °C; assays contained 9% glycerol (w/v) as the viscosogen, which generates the same relative viscosity as that of 100% D_2O at 25 °C (*9*). The amount of glycerol required to achieve a relative viscosity of 1.24 was determined by constructing a standard curve of the viscosity vs percent glycerol (*10*) and determining the required amount of glycerol from the linear standard curve.

pH Studies in the Absence of K^+. To determine whether the kinetic mechanism changes with pH and to obtain an estimate of the K_m values of each of the substrates as a function of pH, initial velocity patterns were measured as a function of pH with the concentration of NAD varied at different fixed levels of MgHIc. The pH-dependence of V and V/K for all substrates in the absence of K^+ was measured under conditions in which one substrate concentration was varied with the other maintained at saturation ($\geq 10K_m$).

The pH was maintained using the following buffers at 100 mM concentration: Bis-Tris, 5.0−6.8; Hepes, 6.8−8.2; Ches, 8.2−10.0. All buffers were titrated to the appropriate pH with tetramethylammonium hydroxide or acetic acid. Sufficient overlap was obtained upon changing buffers to detect buffer effects; none were observed. The ionic strength of the reaction mixtures over the pH range of our measurements is approximately constant at 0.1. The pH was recorded before and after initial velocity data were measured with observed changes limited to ≤0.1 pH unit. The enzyme is stable when incubated for at least 15 min over the pH range 5.0−10.0. pH−rate profiles were initially evaluated graphically for quality of data by plotting log V or log(V/K) against pH.

pH Dependence of Activation Constant of K^+. The pH-dependence of K_{act} for KOAc was obtained under conditions in which the concentration of KOAc was varied with NAD fixed at 0.4 mM and MgHIc maintained at saturated ($10K_m$). The experiment was carried out over the pH range 5.9 − 9.0. Log $1/K_{act}$ (the affinity constant for K^+) was then plotted against pH.

Data Analysis. Initial velocity data were first analyzed graphically using double-reciprocal plots of initial velocities versus substrate concentration and suitable secondary plots. Data were then fitted using the appropriate equation, and the Marquardt−Levenberg algorithm supplied with the EnzFitter program from BIOSOFT, Cambridge, U.K. Kinetic parameters and their corresponding standard errors were estimated using a simple weighting method.

Data obtained on the effect of KCl and KOAc on HIcDH activity were fitted using eq 1. Data obtained with MgHIc and NAD as substrates at different fixed concentrations of KCl were fitted using eq 2. With KOAc as an activator, data were fitted to eqs 3 and 4, which describe fully random and ordered terreactant kinetic mechanisms, respectively. Data obtained using saturating MgHIc and varying NAD and KOAc were fitted using eq 5, which describes a equilibrium ordered mechanism. Data conforming to competitive or noncompetitive inhibition were fitted using eqs 6 and 7. Data for solvent deuterium isotope effects on V and V/K were fitted using eq 8.

$$v = \frac{VA}{K_{act} + A\left(1 + \frac{A}{K_i}\right)} \quad (1)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

$$v = \frac{VABC}{\text{constant} + (\text{coefA})A + (\text{coefB})B + (\text{coefC})C + K_cAB + K_bAC + K_aBC + ABC} \quad (3)$$

$$v = \frac{VABC}{\text{constant} + (\text{coefA})A + 0.3AC + K_aBC + ABC} \quad (4)$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + AB} \quad (5)$$

$$v = \frac{VA}{K_a\left(1 + \frac{I}{K_{is}}\right) + A} \quad (6)$$

$$v = \frac{VA}{K_a\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \quad (7)$$

$$v = \frac{VA}{K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (8)$$

In eqs 1–8, v and V are initial and maximum velocities, A, B, and C are substrate concentrations, I is inhibitor concentration, and K_a, K_b, and K_c are Michaelis constants for substrates A, B, and C, respectively. In eq 1, K_{act} is the activation constant for K^+, and K_i is the inhibition constant for Cl^- or acetate. In eq 2, K_{ia} is the dissociation constant for the EA complex. In eqs 6 and 7, K_{is} and K_{ii} represent inhibition constants for slope and intercept, respectively. In eq 8, F_i is the fraction of D_2O in the solvent, and $E_{V/K}$ and E_V are the solvent deuterium isotope effects minus 1 on V/K and V, respectively.

Data for pH−rate profiles with a limiting slope of 1 at low pH were fitted using eq 9, while data for pH−rate profiles that exhibit a partial change at high pH were fitted using eq 10. Data for pH−rate profiles with a limiting slope of −1 at high pH were fitted using eq 11.

$$\log y = \log\left[C/\left(1 + \frac{H}{K_1}\right)\right] \quad (9)$$

$$\log y = \log\left[\frac{Y_L + Y_H\left(\frac{K_2}{H}\right)}{1 + \frac{K_2}{H}}\right] \quad (10)$$

$$\log y = \log\left[C/\left(1 + \frac{K_2}{H}\right)\right] \quad (11)$$

In eqs 9−11, y is the observed value of the parameter (V, or V/K) at any pH, C is the pH-independent value of y, H is the hydrogen ion concentration, K_1 and K_2 represent acid dissociation constants for enzyme or substrate functional groups important in a given protonation state for optimal binding and/or catalysis, and Y_L and Y_H are the pH-independent values of y at low and high pH, respectively.

RESULTS

Activation of HIcDH by Monovalent Cations. Initial velocity studies were carried out in 20 mM Tris, pH 8.0, to determine the effect of monovalent cations. The reaction rate in the presence of 200 mM (chloride salts) K^+, NH_4^+, Rb^+, Li^+, Cs^+, or Na^+ were measured at fixed concentrations of MgHIc (4.2 μM) and NAD (0.5 mM). The effect of the chloride anion on the reaction rate was also measured under the same conditions. Addition of 200 mM tetramethylammonium chloride caused 64.2% inhibition of the reaction rate compared to the absence of the chloride salts listed above. Elimination of the inhibitory effect of Cl^- gave the following % activation relative to K^+ (for 200 mM salts): K^+ (100%), NH_4^+ (80.9%), Rb^+ (29.3%), and Cs^+ (1.8%). The smaller Li^+ and Na^+ gave 0.5% and 2.3% inhibition, respectively.

Analysis of the Cl^- Inhibitory Effect. Initial rates were measured at varied concentrations of KCl at fixed concentrations of MgHIc (4.2 μM) and NAD (0.5 mM). Data were plotted as shown in Figure 1A, and indicate activation by KCl at low concentration and inhibition above a concentra-

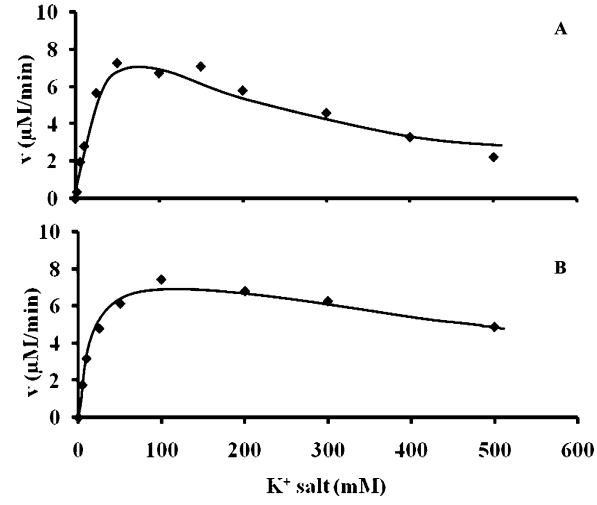


FIGURE 1: Dependence of activity on the concentration of KCl or KOAc. The initial rate was measured at varying concentrations of KCl (A) or KOAc (B) at fixed concentrations of MgHIc (4.2 μM) and NAD (0.5 mM). Data presented in A and B were from a single determination. The points shown are the experimentally determined values, while the curves are theoretical based on fits of the data using eq 1.

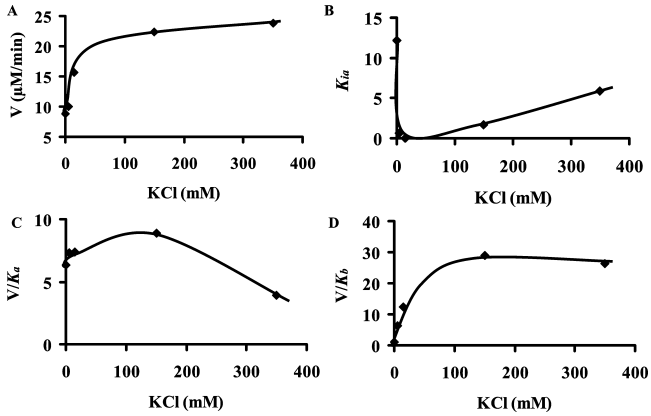


FIGURE 2: Dependence of kinetic parameters on the concentration of KCl. Initial velocity patterns were obtained as a function of MgHIc concentration at different fixed levels of NAD and several fixed concentrations of KCl. Kinetic parameters were then plotted versus KCl concentration. Values of V (A), K_{iMgHIc} (B), V/K_{MgHIc} (C), and V/K_{NAD} (D) obtained from the initial velocity data fitted using eq 2 are shown. Points shown are from fitted data. Units for V, V/K and K_{ia} are μM/min, min^{-1}, and μM, respectively.

Table 1: Dependence of K_{MgHIc} and K_{NAD} on the Concentration of KCl

KCl (mM)	0	5	15	150	350
K_{MgHIc} (μM)	1.4 ± 0.9	1.4 ± 0.5	2.1 ± 0.4	2.5 ± 0.7	6 ± 2
K_{NAD} (mM)	9 ± 3	1.6 ± 0.2	1.3 ± 0.1	0.8 ± 0.1	0.9 ± 0.2

tion of 50 mM. However, inhibition requires higher concentration of KOAc, suggesting anion inhibition, Figure 1B, as suggested above. Data were fitted using eq 1, giving K_{act} values of 55 ± 20 mM and 26 ± 4 mM, respectively for KCl and KOAc, and K_i values of 90 ± 30 mM and 500 ± 100 mM for KCl and KOAc, respectively. The K_{act} values for K^+, whether the chloride or acetate salt was used are equal within error, while the K_i is 5-fold higher when KOAc is used. Data are consistent with K^+ activation and anion inhibition, with the larger Ac^- less effective as an inhibitor.

On the basis of this study, initial velocity patterns were obtained as a function of MgHIc concentration at different fixed levels of NAD and a fixed concentration of KCl. The experiment was then repeated at several different levels of KCl. The data obtained at different KCl concentrations were fitted using eq 2. The kinetic parameters V, V/K_{MgHIc}, V/K_{NAD}, and K_{iMgHIc} were then plotted against KCl concentration and are shown in Figure 2. V/K_{MgHIc} increases and K_{iMgHIc} decreases at low concentrations of KCl, while inhibition is observed with KCl at high concentrations. However, no (or very little) inhibition was observed for V and V/K_{NAD}. Thus, Cl^- exhibits its effect by competing with MgHIc, and binding to either E or E:NAD (*2*). Values of K_{MgHIc} and K_{NAD} in the absence and presence of KCl are listed in Table 1. K_{MgHIc} is similar in the absence and presence of 5 mM KCl, but increases by 5- to 6-fold at 350 mM KCl. On the other hand, K_{NAD} decreases by about 10-fold over the same concentration range.

Overall, data are consistent with an inhibitory effect of chloride. As a result, tetramethylammonium chloride was used to isolate the inhibitory effect of Cl^-. In the absence of K^+, Cl^- is competitive vs MgHIc (K_i = 62 ± 5 mM) and noncompetitive vs NAD (K_{is} = 250 ± 70 mM, K_{ii} = 700 ± 200 mM).

Initial velocity patterns were then repeated for the reaction by varying the concentration of NAD and MgHIc at different fixed concentrations of KOAc. The crossover points for all double-reciprocal plots were to the left of the ordinate (data not shown). All of the initial velocity data were fitted to eq 3 for a fully random terreactant mechanism to determine which terms in the denominator of the rate equation are absent. The K_K term (K_m for K^+) was undefined, which suggests a kinetic mechanism with rapid equilibrium addition of K^+. In addition, the coefNAD and coefK terms were also undefined, suggesting the absence of E−NAD and E−K^+ enzyme forms along the reaction pathway. The K_{NAD} term was also not well defined, but was very well defined in each of the initial velocity pattern obtained at a fixed concentration of KOAc. The value of K_{NAD} was thus fixed as shown in eq 4 and data were fitted to this modified equation with K_K, coefNAD, and coefK eliminated. Values of the remaining kinetic parameters determined were very similar to those obtained using eq 3 and are summarized in Table 2.

Table 2: Kinetic Parameters of HIcDH in the Presence and Absence of KOAc[a]

	plus KOAc	minus KOAc
K_{NAD} (mM)	0.3 ± 0.1	9 ± 3
K_{MgHIc} (μM)	2.0 ± 0.5	1.4 ± 0.9
constant (mM)	14 ± 4	
coefA (mM)	16 ± 3	
K_{iNAD} (mM)		77 ± 57
K_{iMgHIc} (mM)		0.012 ± 0.005
V_1/E_t (s^{-1})	12 ± 1	4.1 ± 0.4
$V_1/K_{NAD}E_t$ (M^{-1} s^{-1})	(4 ± 1) × 10^4	(5 ± 1) × 10^2
$V_1/K_{MgHIc}E_t$ (M^{-1} s^{-1})	(6 ± 1) × 10^6	(3 ± 1) × 10^6

[a] Data were obtained at pH 8.0 and 25 °C.

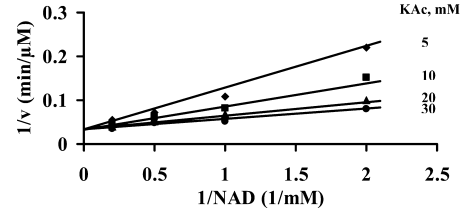


FIGURE 3: Double reciprocal plot obtained with NAD as the variable substrate at different fixed concentrations of K^+. The double reciprocal plot obtained shows the initial rates measured with NAD varied at different fixed concentrations of K^+ and with MgHIc at 20 μM (15K_m). The points are experimental, while the solid lines are theoretical and based on a fit to eq 5.

To test the terreactant kinetic mechanism proposed above, an initial velocity pattern was obtained at varying NAD and different fixed concentrations of KOAc; MgHIc was maintained at a saturating concentration (10K_m). Data are shown in Figure 3. The initial velocity pattern intersects on the ordinate, indicative of the equilibrium ordered addition of K^+ prior to NAD. Estimated values of K_{iK} and K_{NAD} are 43 ± 19 mM and 0.3 ± 0.1 mM, respectively.

Analysis of the Reaction Catalyzed by HIcDH in the Absence of K^+. The initial velocity was measured at different concentrations of NAD and several fixed levels of MgHIc. The double reciprocal plots intersect to the left the ordinate, consistent with the sequential mechanism (data not shown). Data were fitted to eq 2, and kinetic parameters are summarized in Table 2.

Inhibition Studies in the Absence of K^+. On the basis of the Dixon analysis described in Materials and Methods, the K_i for NADH as a product inhibitor is about 2.7 mM. 3-Carboxypropylidenemalate (CPM), a dead-end analogue

Table 3: Solvent Deuterium and Viscosity Effects on Kinetic Parameters in the Absence of K^+

				H_2O/D_2O		H_2O/9% glycerol in H_2O	
	fixed substrates	varied substrate	pH	$^{D2O}(V)$	$^{D2O}(V/K)$	$^{n}(V)$	$^{n}(V/K)$
plus K^+	MgHIc (80 μM)	NAD	8.0	2.6 ± 0.2	1.2 ± 0.2	1.5 ± 0.1	1.6 ± 0.2
	NAD (8 mM)	MgHIc	8.0	2.5 ± 0.2	1.3 ± 0.2	1.5 ± 0.2	0.6 ± 0.1
minus K^+	MgHIc (40 μM)	NAD	8.0	1.7 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.07
	NAD (40 mM)	MgHIc	8.0	1.7 ± 0.1	0.79 ± 0.08	0.9 ± 0.1	0.64 ± 0.09

of HIc, is competitive versus MgHIc with NAD fixed at its K_m; the inhibition constant for CPM is 65 ± 7 μM.

Solvent Deuterium and Viscosity Effects in the Absence of K^+. The pH(D) dependence of kinetic parameters were measured at pH(D) values around 8 to determine whether a solvent isotope effect is observed in the absence of K^+. Data are shown in Table 3. A normal isotope effect was observed on V, while an isotope effect of about unity was observed on V/K_{NAD}. However, the isotope effect on V/K_{MgHIc} was inverse, similar to that observed for the HIcDH catalyzed reaction in the presence of 200 mM KCl. The inverse effect could indicate the presence of increased viscosity of D_2O since η_{rel} is 1.24 in 100% D_2O (9). The inverse effect in the presence of K^+ was interpreted as an effect of viscosity (*7*).

The viscosity effect on kinetic parameters with glycerol as a viscosogen are shown in Table 3. An increase in solvent viscosity has no effect on V and V/K_{NAD}, while an inverse viscosity effect was observed on V/K_{MgHIc}, which is similar to the corresponding solvent isotope effect.

pH Dependence of Kinetic Parameters in the Absence of K^+. The pH dependence of kinetic parameters for HIcDH in the absence of K^+ is shown in Figure 4. V/E_t decreases at low pH with limiting slope of 1, giving a pK of about 6.4. However, no or only a slight decrease was observed at high pH, which differs from the V profile obtained in the presence of 200 mM KCl (*7*). The pH-independent value of V/E_t is 3.6 ± 0.4 s^{-1}, 27.7% of the value measured in the presence of 200 mM KCl. $V/K_{MgHIc}E_t$ is pH independent over the pH range 6.5−7.5 with a pH-independent value of (5.67 ± 0.02) × 10^6 M^{-1} s^{-1}. The $V/K_{MgHIc}E_t$ decreases at low pH with a limiting slope of +2, similar to that observed with HIcDH in the presence of 200 mM KCl. An average pK_a value of about 5.6 was estimated graphically for the two groups, since an equation describing titration of two groups as the pH is decreased is not well conditioned to the data. However, $V/K_{MgHIc}E_t$ also decreases at high pH with limiting slope of −1, giving a pK of 10.6 ± 0.1.

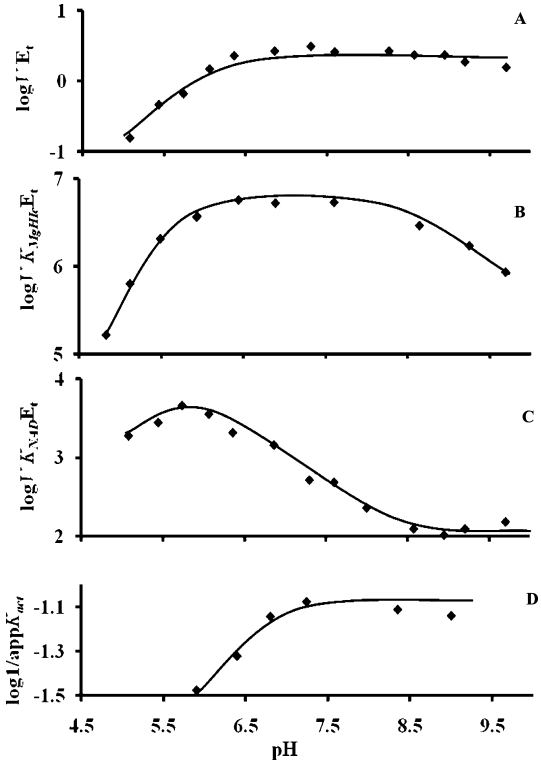


FIGURE 4: pH dependence of the kinetic parameters for the HIcDH. Data for panels A−C were obtained in the absence of K^+. (A) V/E_t (s^{-1}), (B) $V/K_{MgHIc}E_t$ ($M^{-1}s^{-1}$), (C) $V/K_{NAD}E_t$ (M^{-1} s^{-1}), and (D) $1/appK_{act}$ (mM^{-1}). Data were obtained at 25 °C. Points are the experimentally determined values, while the curves are theoretical based on fits of the data to eq 9 for V/E_t, eq 10 for $V/K_{NAD}E_t$, and eq 11 for $V/K_{MgHIc}E_t$. The curve in panel C was drawn by eye.

Solvent Deuterium and Viscosity Effects in the Absence of K^+. The pH(D) dependence of kinetic parameters were measured at pH(D) values around 8 to determine whether a solvent isotope effect is observed in the absence of K^+. Data are shown in Table 3. A normal isotope effect was observed on V, while an isotope effect of about unity was observed on V/K_{NAD}. However, the isotope effect on V/K_{MgHIc} was inverse, similar to that observed for the HIcDH catalyzed reaction in the presence of 200 mM KCl. The inverse effect could indicate the presence of increased viscosity of D_2O since η_{rel} is 1.24 in 100% D_2O (9). The inverse effect in the presence of K^+ was interpreted as an effect of viscosity (*7*).

The viscosity effect on kinetic parameters with glycerol as a viscosogen are shown in Table 3. An increase in solvent viscosity has no effect on V and V/K_{NAD}, while an inverse viscosity effect was observed on V/K_{MgHIc}, which is similar to the corresponding solvent isotope effect.

pH Dependence of Kinetic Parameters in the Absence of K^+. The pH dependence of kinetic parameters for HIcDH in the absence of K^+ is shown in Figure 4. V/E_t decreases at low pH with limiting slope of 1, giving a pK of about 6.4. However, no or only a slight decrease was observed at high pH, which differs from the V profile obtained in the presence of 200 mM KCl (*7*). The pH-independent value of V/E_t is 3.6 ± 0.4 s^{-1}, 27.7% of the value measured in the presence of 200 mM KCl. $V/K_{MgHIc}E_t$ is pH independent over the pH range 6.5−7.5 with a pH-independent value of (5.67 ± 0.02) × 10^6 M^{-1} s^{-1}. The $V/K_{MgHIc}E_t$ decreases at low pH with a limiting slope of +2, similar to that observed with HIcDH in the presence of 200 mM KCl. An average pK_a value of about 5.6 was estimated graphically for the two groups, since an equation describing titration of two groups as the pH is decreased is not well conditioned to the data. However, $V/K_{MgHIc}E_t$ also decreases at high pH with limiting slope of −1, giving a pK of 10.6 ± 0.1.

$V/K_{NAD}E_t$ exhibits behavior similar to that in the presence of K^+, but the pK values of the groups observed in the profile have shifted. $V/K_{NAD}E_t$ decreases at low pH with a limiting slope of 1, while a partial change is observed at high pH. The maximum value is obtained at about pH 5.75, giving a pH-independent value of about (4.2 ± 0.1) × 10^3 M^{-1} s^{-1}. As the pH is increased to 8.5, the rate constant decreases to a constant value of about 123 ± 3 M^{-1} s^{-1}, 2.5% of the value at pH 5.75. The $V/K_{NAD}E_t$ is then pH-independent from pH 8.5 to 9.7.

The pH dependence of the apparent activation constant for K^+ ($appK_{act}$) is shown in Figure 4D. $appK_{act}$ increases below pH 7. A plot of $1/appK_{act}$ vs pH (the affinity constant for K^+) is opposite to that observed for the $V/K_{NAD}E_t$ pH−rate profile, i.e., as the V/K decreases, the affinity for K^+ increases. A pH independent value of about 13 mM is obtained for $appK_{act}$ by averaging the data above pH 7.

DISCUSSION

A comparison of the initial rates of the HIcDH-catalyzed reaction in the presence or absence of 200 mM KCl indicates a 13-fold increase in rate with the concentrations of reactants fixed at their K_m values. With the slow substrate isocitrate (Ic) used as a reactant, the reaction rate was not detectable in the absence of K^+, while it is detectable in the presence of K^+, and 200-fold lower than the rate with HIc (*2*). Data indicate K^+ also activates the reaction with Ic as reactant. Additional studies were not carried out with Ic because of the undetectable rates in the absence of K^+.

The reaction is inhibited at high KCl concentrations, but less inhibition is observed at high concentrations of KOAc, indicating Cl^- is the inhibitor, and not K^+. Tetramethylammonium chloride (TMAC) was used to isolate the inhibition by Cl^-, since the large tetramethylammonium cation exhibits no activation. The initial rate in the presence of 200 mM TMAC is about 36% of that without KCl at fixed concentrations of MgHIc and NAD. All results indicate that Cl^- is the inhibitor of HIcDH activity.

To determine how the chloride ion inhibits the reaction, initial velocity patterns were measured varying MgHIc and NAD at different fixed levels of KCl. Figure 2 shows that K_{iMgHIc} decreases and V/K_{MgHIc} increases at low concentration of KCl, but higher concentrations of K^+ reverse the effect, that is, inhibition occurs. No inhibition of V and V/K_{NAD} was observed. K_{iMgHIc} and V/K_{MgHIc} are obtained at limiting concentrations of MgHIc, while V and V/K_{NAD} are measured at saturating MgHIc; data suggest that Cl^- competes with HIc. Using TMAC as a dead-end inhibitor, competitive inhibition is observed versus MgHIc and noncompetitive inhibition is observed versus NAD, consistent with chloride binding to the HIc binding site.

The value of K_{MgHIc} does not change in the presence of K^+, while K_{NAD} decreases (Table 1). Data likely suggest a decreased affinity for NAD in the absence of K^+, consistent with K^+ binding prior to NAD; the increase in K_{MgHIc} at high concentrations of KCl is a result of chloride inhibition. Consistent with these data, dead-end inhibition by CPM, a competitive inhibitor of HIc, exhibits a K_i similar in the absence and presence of K^+.

To determine the specificity of the activator site, a number of monovalent cations, including NH_4^+, Rb^+, Na^+, Li^+, and Cs^+, were tested. NH_4^+, which has a similar ionic radius to K^+, activates almost as well as K^+. Rb^+ is somewhat larger than K^+, and activates 3-fold less well. Marginal activation was observed for the larger Cs^+, while Li^+ and Na^+ inhibit slightly. It thus appears that K^+ is the best and likely physiologic activator.

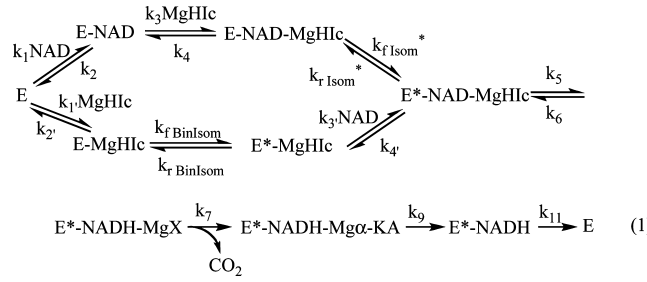
Kinetic Mechanism in the Presence of KOAc. Data were obtained with KOAc as an activator to take advantage of the much higher inhibition constant for acetate. K^+ was considered a pseudosubstrate, and a terreactant analysis of initial rates was conducted as for KCl. As suggested in the Results section, data are consistent with a fully ordered mechanism with the binding of MgHIc followed by K^+ and NAD, and with K^+ binding in rapid equilibrium.

With KCl as an activator, the kinetic mechanism for addition of MgHIc and NAD is random, and the pathway with MgHIc adding prior to NAD is preferred (*2*). The change to a fully ordered mechanism in the presence of KOAc is likely due to elimination of the inhibitory effect of chloride ion. Chloride decreases the apparent affinity for MgHIc, and allows the binding of NAD to free enzyme to be observed.

Influence of K^+ Ion on Solvent Deuterium and Viscosity Effects in the Absence of K^+. In the presence of K^+, a significant viscosity effect is observed on V and V/K_{NAD}. In the absence of K^+, the viscosity effect on V and V/K_{NAD} decreased to 1, suggesting diffusion-limiting steps, such as product release or NAD binding do not contribute to rate limitation in the absence of K^+. However, a similar inverse viscosity effect was observed on V/K_{MgHIc} in the absence or presence of K^+. Thus, whether or not K^+ is present, the viscosity effect on V/K_{MgHIc} likely reflects stabilization of an enzyme form that exists when MgHIc binds (*7*).

The solvent deuterium isotope effects observed in the absence of K^+ are similar to those obtained in the presence of K^+. Proton transfer step(s) still contribute to the rate limitation of the overall reaction in the absence of K^+. A normal isotope effect is observed on V, suggesting a proton transfer step contributes to rate limitation at saturating conditions. The normal SKIE on V is lower in the absence than in the presence of K^+, and the solvent viscosity effect has decreased from 1.5 to 1. Data suggest that the diffusion-related step that contributed to rate-limitation at saturating reactant concentrations no longer contributes. In addition, the proton transfer step that contributes to rate-limitation in the absence of K^+ must be an isomerization of E, E−NAD or E−MgHIc, since there is no significant SKIE on V/K_{NAD} or V/K_{MgHIc}, as discussed below. A $^{D2O}(V/K_{NAD})$ of 1, within error, is observed. Although the SKIE on V/K_{MgHIc} is inverse, it likely reflects the inverse solvent viscosity effect on V/K_{MgHIc}, Table 3, as discussed above. Subtracting the observed solvent viscosity effect gives an SKIE of about 1 on V/K_{MgHIc}.

Data obtained in the absence of K^+ help to clarify the isotope and viscosity effects observed for HIcDH in the absence and presence of K^+. Effects other than unity observed in the absence of K^+ are the SKIE of 1.7 on V, and the inverse SKIE and viscosity effect on V/K_{MgHIc}. The HIcDH has a steady state random kinetic mechanism (*2*), which can be modified to accommodate the data obtained in these studies. Consider mechanism 1:



In mechanism 1, k_1, k_2, k_3 and k_4 are binding and dissociation rate constants for the pathway with NAD binding before MgHIc, while k_1', k_2', k_3' and k_4' reflect the bottom pathway with MgHIc binding first. Note there are two isomerizations allowed upon binding of MgHIc. Isomerization of the binary E−MgHIc complex is represented by $k_{f\,BinIsom}$ and $k_{r\,BinIsom}$, and may contribute to the expression for V, but will not contribute to the expressions for the V/Ks, while isomerization of the ternary E−NAD−MgHIc complex is given by $k_{f\,Isom}$* and $k_{r\,Isom}$*, and could contribute to the expressions for V and V/K_{MgHIc}. The oxidation step is given by k_5 and k_6, decarboxylation and rapid release of CO_2 has rate constant k_7, while k_9 and k_{11} represent release of the products α-KA and NADH. It is the isomerization steps that are likely responsible for the isotope and viscosity effects, as suggested for enzyme in the presence of 200 mM KCl (*2*). Thus, the isomerized form of the E−NAD−MgHIc complex is stabilized in the presence of viscosogen, while isomerization of the binary E−MgHIc complex is responsible for the SKIE. The proton dependence of the binary complex isomerization suggests that a proton is either released or taken up as MgHIc binds. This is apparently not true for the ternary complex isomerization, suggesting NAD binding either eliminates the proton involved or locks it on enzyme. This will have to be sorted out in future studies. Data further indicate that the SKIE on V must be a combined effect that includes both the normal SKIE and the inverse viscosity effect, suggesting the SKIE on binary complex isomerization corrected for the viscosity effect is closer to 2.8 (1.7/0.6, Table 3).

The normal viscosity effect of about 1.5 observed on V and V/K_{NAD} observed for enzyme in the presence of 200 mM KCl

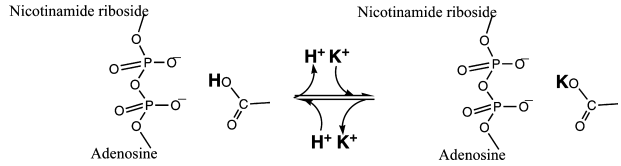


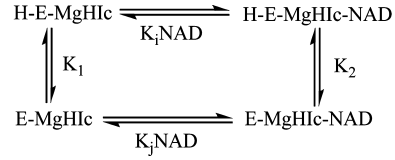
FIGURE 5: Proposed mechanism of potassium activation of HIcDH. Potassium neutralizes a negative charge that is created as a group on enzyme is titrated.

must reflect isomerization of E−MgHIc and binding of NAD (as suggested previously (*7*)), respectively. Elimination of K^+, which must bind prior to NAD, and Cl^-, which competes with NAD, results in an increase in the rate of binding NAD relative to other steps along the reaction pathway.

pH Studies in the Absence of K$^+$. In the presence of K^+, the *V* pH−rate profile exhibits a hollow on the acid side with a pK_a of about 7 and a pK_a of about 9.5 on the basic side (*7*). The hollow was interpreted in terms of stickiness of the proton and MgHIc in the H−E−NAD−MgHIc complex. The pK_a on the basic side was attributed to the general acid-catalyzed tautomerization of the enol of α-ketoadipate, suggesting that, in the absence of K^+, other steps become rate-limiting. The *V* pH−rate profile is much simpler in the absence of K^+. *V* decreases at low pH with a pK_a of 6.4, likely the same group observed in the *V* profile in the presence of K^+. No hollow was observed, which suggests the proton, substrate, or both are no longer sticky in the H−E−NAD−MgHIc complex. In addition, no pH dependence is observed on the basic side of the profile.

The V/K_{MgHIc} pH−rate profile exhibits a slope of 2 on the acid side in the absence and presence of K^+. One of the two groups is likely the third pK_a of HIc, while the second is likely the general base that accepts a proton from the 3-hydroxyl of HIc in the hydride transfer step, as suggested for the presence of K^+. The average pK_a, obtained graphically, is about 5.6. In the absence of K^+, however, V/K_{MgHIc} decreases at high pH with a pK_a of around 10, that is not observed in the presence of K^+. Since this group is not observed in the *V* profile, it is likely important for binding of HIc. In the presence of K^+, the pK_a is likely >10.

The $V/K_{NAD}E_t$ pH−rate profile, in the presence of K^+, exhibits a group with pK_a of 6.4 at low pH, which likely reflects the general base in the E−MgHIc complex. A partial change in the second order rate constant was observed at high pH giving a pK_a of about 8.3, and an approximately 4-fold decrease in the rate constant (*7*). In the absence of K^+, the V/K_{NAD} pH−rate profile exhibits a qualitatively similar shape, but the pK_a for both groups are apparently shifted to lower pH. However, the pK_a value estimated from the changes observed at high and low pH are very close together, and thus the true pK_a values cannot be estimated, rather an average that is equal to the pH at the maximum value of *V*/*K*, i.e., ∼pH 6. The decrease in V/K_{NAD} observed at low pH likely reflects the general base as it does in the presence of K^+. The partial change observed in the V/K_{NAD} pH−rate profile in the absence of K^+, results in a decrease of >30-fold in the rate constant. Since this partial change is not observed in the *V* profile, it must reflect a decrease in affinity of enzyme for NAD as a group on enzyme is titrated. The affinity of enzyme for NADH also decreases given the K_i of 0.3 mM measured in the presence of K^+ (*2*), and the value of ∼2.7 mM measured in the absence of K^+ (see Results). The high value of K_{NAD} argues it is a K_d value. Given the decreased affinity of HIcDH for NAD and NADH, the group on enzyme that gives the decreased affinity when titrated may be a neutral acid such as aspartate or glutamate in the vicinity of the pyrophosphoryl moiety of the dinucleotides. The partial change can be described using the thermodynamic cycle in mechanism 2. Calculated K_{iNAD} and



(2)

K_{jNAD} values (from the ratio of *V* and *V*/*K*) gives estimates 0.9 and 30 mM, respectively, and a group with a pK_1 of about 6 in the absence of NAD has a pK_2 of about 7.5 when NAD is bound.

In the presence of K^+ the decrease in affinity is not observed until the pH is greater than 8, indicating that the monovalent ion can take the place of the proton. This is clearly the biggest effect of K^+, i.e., to facilitate the binding of NAD at high pH, when the enzyme group has become unprotonated. All other effects of K^+ are likely related to the effect on NAD binding. Consistent with this suggestion, the affinity constant for the monovalent ion, Figure 4D, is pH independent above pH 7, and its affinity decreases as the group on enzyme becomes protonated. Data suggest K^+ takes the place of the proton on the group that must be protonated for the binding of NAD. They are thus located at the same site and most likely bind to the pyrophosphoryl group of NAD, Figure 5. On the basis of these results, the loss in affinity for NAD observed in V/K_{NAD} pH−rate profile at high pH would be eliminated in the presence of K^+ and the absence of Cl^-.

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