Regulatory Mechanism of Histidine-tagged Homocitrate Synthase from *Saccharomyces cerevisiae*

II. THEORY

In this study, rate equations that predict the regulatory kinetic behavior of homocitrate synthase were derived, and simulation of the predicted behavior was carried out over a range of values for the kinetic parameters. The data obtained allow application of the resulting expressions to enzyme systems that exhibit activation and inhibition as a result of the interaction of effectors at multiple sites in the free enzyme. Homocitrate synthase was used as an example in terms of its activation by Na\(^+\) binding to the active enzyme conformer at an allosteric site, inhibition by binding to the inactive site, and activation by lysine binding to the less active enzyme conformer.

Homocitrate synthase (HCS\(^2\), EC 2.3.3.14, 2-hydroxybutane-1,2,4-tricarboxylate-2-oxoglutarate-lyase (CoA-acetylating)) catalyzes the first and committed reaction in the 2-oxoglutarate cycle in yeast (8). It has also been reported that HCS activity can be regulated by lysine (5). It has also been reported that HCS activity can be higher in fungi (1–4). Both cytosolic and mitochondrial isozymes of HCS from *Saccharomyces cerevisiae* are feedback-inhibited by lysine (5). This paper is available online at http://www.jbc.org.

**EXPERIMENTAL PROCEDURES**

Data Analysis and Simulation—The kinetic parameters used for simulation of kinetic behavior were obtained by fitting the experimental data presented in the accompanying article (11) using the appropriate rate equations and the Marquardt-Levenberg algorithm supplied with the Enzfitter program from Biosoft (Cambridge, UK). All of the simulations were carried out using the appropriate derived equations and Mathematica Version 5.0.1.0 (Wolfram Research, Inc., Champaign, IL).

**Theory**—On the basis of the experimental data presented in the accompanying article (11), a schematic of the regulatory kinetic mechanism of HCS by Na\(^+\) is shown in Scheme 1. The model is general in that it allows for binding of the effector to all enzyme forms. As described previously (9), a conformational equilibrium exists for HCS in the absence of reactants. The activation, effected by the cations, can be explained by stabilizing the E (active) form of HCS. In the case of HCS, Scheme 1 is reduced to a consideration of the effector stabilizing only the E form, with no effect observed as reactants bind (see accompanying article (11)). The active form of the free enzyme is also subject to inhibition by the same effector that activates the enzyme. This phenomenon is separate from activation. In deriving the equation, all steps for binding of Na\(^+\) and the enzyme conformational change were assumed to be at equilibrium as shown in Scheme 1, and the mechanism is simplified as at the bottom of Scheme 1 (8). The simplified scheme was then used to derive the rate equation by the King-Altman method (7).

The fractions of productive enzyme, *i.e.* enzyme forms that go on to give product, at equilibrium with X are given as follows. Productive enzyme fractions are defined as \(f_{E_{\text{AB}}} = \frac{E_{\text{AB}}}{X}\) and \(f_{E_{\text{AB}}^*} = \frac{E_{\text{AB}}^*}{X}\), where Equation 1 follows.

\[
X = E + E\text{Na}^+ + E' + E\text{Na}' + EA + E\text{Na}^+A + EB + E'B + E\text{Na}'B
\]

\[+ E'\text{Na}'B + EAB + E\text{Na}'AB + Na'E + Na'E\text{Na}' (\text{Eq. 1})\]

Equilibrium dissociation constants can be written for each step and can be used to solve for the concentration of species in terms of effector concentration, the dissociation constant, and the concentration of E. Thus, \(E\text{Na}' = E\text{Na}'/K_{\text{Na}'}, E = E/K_{\text{eff}}, E\text{Na}^+ = E\text{Na}^+/K_{\text{Na}^+} (K_{\text{Na}^+}), Ea = E/A/K_{\text{Na}^+}, E\text{Na}^+A = E\text{Na}^+/K_{\text{Na}^+}A(K_{\text{Na}^+}), E'B = E/B/K_{\text{Na}'}, E' = E'/K_{\text{Na}'}, E\text{Na}'B = E\text{Na}'/K_{\text{Na}'} (K_{\text{Na}'}, EAB = E/A/K_{\text{Na}'+}(A/K_{\text{Na}'}K_{\text{Na}'}B/K_{\text{Na}'}, Na'E = E\text{Na}'/K_{\text{Na}'}, and Na'E\text{Na}' = E\text{Na}'(K_{\text{Na}'}/K_{\text{Na}'}, f_{E_{\text{AB}}} = \frac{E_{\text{AB}}}{X}\) is given in Equation 2.

\[
f_{E_{\text{AB}}} = \frac{EAB}{E + EA + E\text{Na}'A + EB + E\text{Na}'B + EAB + E\text{Na}'AB + E' + E'B + E\text{Na}'B + E\text{Na}'E + Na'E\text{Na}' + Na'E} (\text{Eq. 2})
\]

Substitution of each of the enzyme forms in Equation 2 with the parameters defined above gives Equation 3.

\[
f_{E_{\text{AB}}} = \frac{K_{\text{Na}'}K_{\text{Na}'+} + (K_{\text{Na}'}A + K_{\text{Na}'+}B/K_{\text{Na}'}) + AB}(1 + \frac{Na'}{K_{\text{Na}'}}) + \frac{K_{\text{Na}'K_{\text{Na}'}B/K_{\text{Na}'}}}{K_{\text{Na}'}} (\text{Eq. 3})
\]

Equation 3 can be rearranged as shown in Equation 4.
Simulation of Regulatory Mechanism of Homocitrate Synthase

Scheme 1. Upper, kinetic mechanism for activation by Na$^+$, A, B, P, Q, Na, E, and E represent α-Kg, AcCoA, CoA, homocitrate, Na$^+$, and the active and less active forms of the enzyme, respectively. $K_{act}$ is the equilibrium constant between the free enzyme in the active and less active forms, whereas $K_{act}'$ is the same conformational equilibrium in the presence of Na$^+$. $K_{act}$ and $K_{act}'$ are the activation constants of Na$^+$ for the active and less active conformers, respectively. Lower, simplification of the Na$^+$ activation model using the equilibrium assumption of Cha (8). According to the King-Altman algorithm (7), the simplified model contains three major enzyme-containing species represented as X, Y, and Z. Each major species of the enzyme contains other enzyme subspecies in equilibrium. In the simplified model, $f_j$ (J = enzyme forms) is the fraction of each enzyme form that contributes to the overall catalytic rate of the next major enzyme species.

$$f_{EAB} = \frac{AB}{K_{act}K_n\left[1 + K_{act}'\left(\frac{Na}{K_{act}}\right)\right] + \left(1 + K_{act}'\left(\frac{Na}{K_{act}}\right)\right)B + (K_{eq}A + AB)\left(1 + \frac{Na}{K_{act}}\right)}$$

(Eq. 4)

$f_{EAB}'$ can be defined as shown in Equation 5.

$$f_{EAB}' = f_{EAB}\left(\frac{Na}{K_{act}}\right)$$

(Eq. 5)

All of the other fractions ($f_j$) can be defined in the same way for Y and Z (Scheme 1). Therefore, the ENaPQ (where P and Q are AcO and homocitrate, respectively) to EPQ and ENaQ to EQ equilibria are included in Y and Z, respectively. However, as long as the affinity for the effector is independent of bound reactants, $f_{EAB} = f_{EAB} = 1/(1 + (Na/K_{act}))$ and $f_{P/Q} = f_{P/Q} = (Na/K_{act})(1 + (Na/K_{act}))$. The expression for $E_i$ (total enzyme) is equal to the sum of all enzyme forms (i.e. X, Y, and Z), and this gives the denominator of the final rate equation. X, Y, Z, and $v$ (initial rate) can be derived as follows, where $\Delta$ is the sum of all of the numerator terms in X, Y, and Z (Equations 6–9).

$$X = \frac{(h_2'Na_{K_{act}} + h_3)(h_1'Na_{K_{act}} + h_11)}{\Delta}$$

(Eq. 6)

$$Y = \frac{(h_2'Na_{K_{act}} + h_3)(h_1'Na_{K_{act}} + h_11)}{\Delta}$$

(Eq. 7)

$$Z = \frac{(h_2'Na_{K_{act}} + h_3)(h_1'Na_{K_{act}} + h_11)}{\Delta}$$

(Eq. 8)

Substitution of the expression for $\Delta$ in Equation 9 gives Equation 10.

$$v = \frac{(h_2'Na_{K_{act}} + h_3)(h_1'Na_{K_{act}} + h_11)}{\Delta}$$

(Eq. 9)

Substitution of the expression for $f_{EAB}$ into Equation 10 gives Equation 11.

In Equation 11, if $h_j = h_j'$ (microscopic rate constants for binding and catalytic steps), as is true for Na$^+$ activation of HCS, then the equation reduces to Equation 12 after rearrangement.
\[ V = \frac{KA}{k + kA + AB} \]

Equation 14.

\[ V = \frac{v}{K + v} \]

\[ v = 0 \]

\[ V = \frac{1 + Na^{+}}{K + Na^{+}} \]

Equation 13.

\[ V = \frac{1 + Na^{+}}{K + Na^{+}} \]

Conditions

| Conditions | A (low), B (low) | A (high), B (low) |
|------------|-----------------|------------------|
| Na\(^{+}\) = 0 | \[ V = \frac{KA}{k + kA + AB} \] | \[ V = \frac{v}{K + v} \] |
| K\(_{i}\) > Na\(^{+}\) > K\(_{act}\) | \[ V = \frac{1 + Na^{+}}{K + Na^{+}} \] | \[ v = 0 \] |
| Na\(^{+}\) >> K\(_{i}\), K\(_{act}\) | \[ V = \frac{1 + Na^{+}}{K + Na^{+}} \] | \[ v = 0 \] |

The mechanism in the absence of Na\(^{+}\) is sequential ordered (10). Thus, expressions for kinetic parameters can be generated from Equation 12 at the limit where the Na\(^{+}\) concentration is zero, as shown in Equation 13.

\[ V = \frac{(k_{b}k_{11})(1 + Na^{+})ABE_{i}}{K_{a}K_{b}(k_{b}k_{11})(1 + K_{act}) + K_{a}(k_{b}k_{11})(1 + K_{act})B + K_{a}(k_{b}k_{11})A + (k_{b}k_{9} + k_{b}k_{11} + k_{b}k_{11})AB} \]

Dividing the numerator and denominator of Equation 13 by coefficient AB gives Equation 14.

\[ V = \frac{(k_{b}k_{11})ABE_{i}}{K_{a}(k_{b}k_{11})(1 + K_{act}) + K_{a}(k_{b}k_{11})(1 + K_{act})B + K_{a}(k_{b}k_{11})A + (k_{b}k_{9} + k_{b}k_{11} + k_{b}k_{11})AB} \]

In Equation 16, for HCS, \( K_{i}, K_{act}', \) and \( K_{act}'' \) are the dissociation constant for \( \alpha \)-ketoglutarate (\( \alpha \)-Kg) and the Michaelis constants for \( \alpha \)-Kg and AcCoA, respectively; A and B represent the concentrations of \( \alpha \)-Kg and AcCoA, respectively; \( K_{act}, K_{act}', \) and \( K_{act}'' \) reflect the activation constant for Na\(^{+}\), the conformational equilibrium between \( E \) and \( E' \) in the presence of Na\(^{+}\), and the inhibition constant for Na\(^{+}\), respectively; \( K_{act}'' \)
is the equilibrium constant between E and E' in the absence of Na'. and 
Na' is the Na' concentration. Table 1 gives the limits of Equation 16.

In the presence of both lysine and Na', the behavior of the enzyme is
more complicated because of the simultaneous activation and inhibition
effects of Na' and inhibition by lysine. The kinetic mechanism in this case
is shown in Scheme 2. However, there is no experimental evidence to
suggest that any lysine-bound E form of the enzyme can be formed (see
accompanying article (11)). In this case, all of the enzyme species shown
are assumed to be at equilibrium, and the rate of the enzyme reaction
depicted in Scheme 2 is represented as $v = k_p(E + ENa')/E)$. $k_p$ is the
overall enzymatic rate in the absence of inhibitors (not $V$ because A and B
are maintained at a fixed low concentration) and activators. $E$ is the sum
of all enzyme species that are in equilibrium. The rate equation for
prediction of enzyme behavior (derived as described above) in this case is
as follows (Equation 17),

$$
\frac{1}{v} = \frac{k_p \left(1 + \frac{Na'}{K_{act}}\right)}{1 + K_{act}(1 + \frac{1}{1 + \frac{Na'}{K_{act}}})},
$$

(Eq. 17)

where $K_{act}$ is the activation constant of Na' for the E'-Lys complex.
When Na' and Lys are at zero concentration, $v$ is equal to $k_p/(1 + K_{act})$.

RESULTS AND DISCUSSION

The effect of Na' on HCS activity is complex, with activation
and inhibition occurring simultaneously depending on the con-

**Fig. 1.** Tertiary replot of the SOS for the inhibition and activation by Na'. $x_1$, $x_2$, and $x_3$ represent $K_{act}$, $K_i$, respectively. Values of $x$ and $y$ are defined under “Results and Discussion.”

The double-reciprocal plot of Equation 16 with the AcCoA concentration varied at fixed levels of a-Kg is given in Equation 18.

$$
\frac{1}{v} = \left(\frac{K_{act}K_{act}'}{V}\right)(1 + K_{act}) + \left(\frac{1}{1 + \frac{Na'}{K_{act}}}\right)\left(\frac{1}{1 + \frac{Na'}{K_{act}}}\right)\left(\frac{1}{A + \frac{1}{V}}\right)
$$

(Eq. 18)

**Fig. 2.** Tertiary replot of the SOI for the inhibition and activation by Na'. The values of $x$ and $y$ are defined under “Results and Discussion.”

On the basis of Equation 18, expressions for the slope and intercept are given in Equations 19 and 20.
If $K_{\text{conf}}'$ is equal to zero, as found experimentally for HCS, then on the basis of Equations 19 and 20, expressions for the slope of the slope (SOS) and the slope of the intercept (SOI) are given in Equations 21 and 22.

**SOS**

$$\text{SOS} = \left( \frac{K_v K_a'}{V} \right) \left( 1 + \frac{K_{\text{conf}}}{1 + \frac{N_o^-}{K_{\text{act}}} + \left( \frac{N_o^-}{K_{\text{act}}} \right)^2} \right) + \frac{1}{V} \left( 1 + \frac{N_o^-}{K_{\text{act}}} \right) \left( \frac{N_o^-}{K_{\text{act}}} \right)^2$$

(Eq. 21)

**SOI**

$$\text{SOI} = \left( \frac{K_v}{V} \right) \left( 1 + \frac{K_{\text{conf}}}{1 + \frac{N_o^-}{K_{\text{act}}} + \left( \frac{N_o^-}{K_{\text{act}}} \right)^2} \right) + \frac{1}{V} \left( 1 + \frac{N_o^-}{K_{\text{act}}} \right) \left( \frac{N_o^-}{K_{\text{act}}} \right)^2$$

(Eq. 22)

Tertiary replots are given by the slope of the secondary plot (slope versus 1/o-Kg) or the SOS versus Na+ concentration (Equation 21) and the intercept of the secondary plot (intercept versus 1/o-Kg) or the SOI versus Na+ concentration (Equation 22) and are shown in Figs. 1 and 2. The SOS/SOI ratio is given by Equation 23 and is equal to the effective $K_i$ for Na+ at the AcCoA site.

$$\text{SOS/SOI} = \left( \frac{K_v K_a'}{K_v} \right) \left( 1 + \frac{1}{1 + \frac{N_o^-}{K_{\text{act}}} + \left( \frac{N_o^-}{K_{\text{act}}} \right)^2} \right)$$

(Eq. 23)

As shown in Figs. 1 and 2, the y intercepts ($y_j$) represent the SOS and SOI at zero NaCl, respectively, given by $y_{1-\text{SOS}} = (K_v K_a' / V)(1 + K_{\text{conf}})$ and $y_{1-\text{SOI}} = (K_v / V)(1 + K_{\text{conf}})$.
The point marked $x_2$ in the SOS and SOI tertiary replots is equal to $K_{\text{conf}}$, i.e. the concentration of Na$^+$ that gives half-maximum activation in the absence of Na$^-$ inhibition. In Fig. 1, $y_2$ is the maximum activity for the enzyme in the absence of Na$^-$ inhibition. The value of $y_2$ is $K_{\text{conf}}/V$ (obtained from the $y$ intercept of Equation 21 when Na$^-$ tends to infinity in the absence of Na$^+$ inhibition).

The value of $y_2$ in the SOI plot is $K_v/V$ (Fig. 2), obtained from Equation 22 at infinite Na$^+$. In both SOS and SOI plots, the $y_2/y_1$ ratio is $1 + K_{\text{conf}}$ (the fraction of activated enzyme). In Fig. 1, $x_2$ is $K_1$ for Na$^-$ inhibition for the fully activated enzyme. If $K_1$ goes to infinity (high AcCoA), then the value of $x_1$ (obtained using the derivative of Equation 21) in Fig. 1 (Equation 24) is comparable with the value of $x_1$ in Fig. 2 (Equation 25), where $x_1$ is an apparent $K_{\text{act}}$ value that depends on the values of $K_{\text{conf}}$ and $K_1$. In other words, it determines the intrinsic effects of Na$^+$ inhibition and conformational changes on the $K_{\text{act}}$ value. In the case of HCS, the effect is negligible. If $K_{\text{act}}$ goes to infinity and $K_{\text{conf}}$ goes to zero, then the value of Equation 24 is $-K_1$.

$$x_\text{1-sos} = \frac{1}{1 + K_{\text{conf}}(K_{\text{conf}} - 1/K_1)} \quad \text{(Eq. 24)}$$

$$x_\text{1-soi} = K_{\text{act}}(1 + 1/K_1) \quad \text{(Eq. 25)}$$

As Na$^+$ goes to infinity in Equation 21, the equation will reduce to $(K_vK_1/V)(1 + (Na^-/K_v))$, which is the equation for the dotted line that extrapolates to $x_2$ as the SOS goes to zero. If there is no activation by Na$^-$, Equation 21 is equal to $(K_vK_1/V)(1 + K_{\text{conf}} + (Na^-/K_v))$. In this case, the experimentally measured $K_v$ is equal to $K_1(1 + K_{\text{conf}})$. Between the two extremes, no activation as a result of an infinite $K_{\text{act}}$ and completely activated enzyme as a result of infinite Na$^-$, a series of parallel lines is obtained for a plot of the SOS versus Na$^-$ concentration at constant $K_v$. The $y$ intercept depends on the value of $K_{\text{conf}}$. As the $K_v$ for inhibition becomes greater than the $K_{\text{act}}$ for activation by Na$^-$, then the SOS plot (Fig. 1) will show a lower minimum value, indicating a greater observed activation, and the linear part of the curve will have a greater slope as Na$^-$ is increased.

Data simulation using the experimentally measured kinetic parameters (see accompanying article (11)) shows the dependence of activation on $K_{\text{act}}$. If the $K_{\text{act}}$ for Na$^-$ is much greater than the $K_1$ (37 mM), then as described in Equation 21, the SOS versus Na$^-$ concentration will approach linearity (Fig. 3A). If the $K_1$ increases, the plot shows less and less inhibition (Fig. 3B). A three-dimensional plot of the above is shown in Fig. 3C.

The SOI (Fig. 4) represents a measure of enzyme activity at high AcCoA and low alk-Kg and exhibits no inhibition by Na$^-$. In other words, an infinite concentration of AcCoA eliminates the Na$^-$ inhibition by increasing its $K_v$ to infinity. The percentage of HCS enzyme activity versus Na$^-$ concentration based on the derived equations is in very good agreement with the experimental data (see accompanying article (11)), indicating that the equations precisely predict the enzyme behavior of HCS at different Na$^-$ concentrations (Fig. 5). Calculation of the percentage of HCS activity is derived by dividing Equation 16 by Equation 16 when the Na$^-$ concentration is zero, with the assumption that $K_{\text{conf}} = 0$, and is shown in Equation 26.

$$\% \text{ activity} = \frac{[K_vK_1 + K_vB + (K_vA + AB)](1 + Na^-)}{K_vK_1(1 + K_{\text{conf}} + (Na^-/K_v))B + (K_vA + AB)(1 + Na^-)} \times 100 \quad \text{(Eq. 26)}$$

As shown in Fig. 5 (A and C), increasing the alk-Kg concentration decreased both the activatory and inhibitory effects at all levels of Na$^-$. Interestingly, the common point for all of the
curves in Fig. 5C is 150 mM Na\(^+\), where the HCS activity remains at 100%, and this has physiological significance (see accompanying article (11)). In terms of Equation 26, increasing the level of substrate A gives 100% activity. However, increasing the AcCoA concentration (B goes to infinity) only eliminated the inhibitory effects of Na\(^+\), whereas it had no effect on the activation by Na\(^+\) (Fig. 5, B and D). The same mechanism can also be used to explain the behavior of other monovalent or divalent cations (see accompanying article (11)).

The effects of lysine and Na\(^+\) in the absence and presence of AcCoA are complex (see accompanying article (11)). The equation predicting enzyme activity in this case is as follows, with the assumption that \(K_{\text{conf}} = 0\) (see accompanying article (11)) (Equation 27),

\[
\frac{1}{v} = \left( \frac{K_{\text{act}} (1 + K_{\text{act}}) + (1 + K_{\text{act}}) \left( \frac{Na^+}{K_{\text{act}}} \right) Lys}{k_a K_{\text{Lys}} (1 + Na^+ \frac{K_{\text{act}}}{K_{\text{act}}})} \right)
\]

where Na\(^+\), Lys, \(K_a\), and \(K_{\text{Lys}}\) are the concentrations of Na\(^+\) and lysine and the inhibition constants for Na\(^+\) and lysine, respectively, and \(k_a(1 + K_{\text{conf}})\) is the overall enzymatic rate in the absence of inhibitors and activators at fixed concentrations of α-Kg and AcCoA. The slope of the above equation when \(1/v\) is plotted versus lysine concentration represents the enzyme activity as lysine tends to infinity. This equation can be used to explain HCS behavior in the presence of Na\(^+\) and lysine at both low and high AcCoA concentrations. However, the \(K_a\) and \(K_{\text{act}}\) values differ at high and low AcCoA concentrations. As shown in Fig. 6, the slope of Equation 27 versus Na\(^+\) concentration can be concave upward or downward depending on the values of \(K_{\text{act}}\) and \(K_{\text{act}}^\prime\). However, the values of \(K_{\text{act}}\) and \(K_{\text{act}}^\prime\) affect the slope in opposite directions, and concentrations higher than 150 mM Na\(^+\) (in the case of HCS) had almost no effect on the slope either at low or high AcCoA concentration. As mentioned above, this phenomenon is thought to have physiological significance (see accompanying article (11)). Fig. 7 shows the intercept of Equation 27, i.e. when the lysine concentration is zero. In this case, the equation reflects the kinetic behavior of the enzyme in the presence of Na\(^+\) as expected, and the intercept effect depends on the values of \(K_{\text{act}}\) and \(K_a\). Increasing the \(K_{\text{act}}\) value eliminated the Na\(^+\) activation only and had little effect on the intercept values (low or high AcCoA concentration) (Fig. 7A). However, changing
the $K_v$ value dramatically changed the intercept (low and high AcCoA concentrations). Na$^+$ inhibition was eliminated at higher $K_v$ values, whereas activation remained.

In conclusion, the regulatory kinetic behavior of homocitrate synthase demonstrates the effect of a bifunctional activatory/inhibitory effector. In this context, the enzyme simultaneously senses the activatory and inhibitory effects of the effector, and the net catalytic outcome depends on the concentration of the effector and the activation and inhibition constants. The resulting expressions can be applied to enzyme systems that exhibit activation and inhibition as a result of interaction at different sites in the free enzyme.

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