Kinetic and Regulatory Mechanisms for (Escherichia coli) Homoserine Dehydrogenase-I

EQUILIBRIUM ISOTOPE EXCHANGE KINETICS*

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Isotope exchange kinetics at chemical equilibrium were used to probe the mechanisms of substrate binding and regulatory behavior of homoserine dehydrogenase-I from Escherichia coli. At pH 9.0, 37 °C, $K_{m} = 100 \pm 20$ for the catalyzed reaction: L-aspartate-$\beta$-semialdehyde + NADPH + $H^{+} = L$-homoserine + NADP$. Saturation curves for the exchange reactions, $[^{14}C]_L$-homoserine $\rightleftharpoons L$-aspartate-$\beta$-semialdehyde and $[^{3}H]_N$ADPH $\rightleftharpoons$ NADPH were observed as a function of different reactant-product pairs, varied in constant ratio at equilibrium. The NADP$^+$ $\rightleftharpoons$ NADPH exchange rate was inhibited upon variation of pairs involving L-aspartate-$\beta$-semialdehyde and L-homoserine, consistent with preferred order random binding of cofactors before amino acids. Optimal rate constants, derived by simulations of equilibrium isotope exchange kinetics data with the ISOBI program, indicate faster dissociation of amino acids than cofactors from the central complexes but nearly equal rates for association of cofactors and amino acids to free enzyme. Rate limitation of net turnover in both directions is determined by dissociation of cofactor from the $E$-cofactor complex. The allosteric modifier, L-threonine, produces distinctive perturbations of the saturation curves for isotope exchange, which were analyzed systematically with the ISOBI program. The best fit to the data was obtained by L-threonine inhibiting catalysis between the central complexes without altering substrate association-dissociation rates. Simulations also showed that rate-limiting catalysis suppresses the kinetic inhibition effects that are characteristic of preferred order substrate binding, producing patterns typical for a (rapid equilibrium) random kinetic scheme.

Aspartokinase (AK), the first enzyme in the pathway leading from L-aspartate to L-homoserine, L-threonine, L-lysine, L-isoleucine, and L-methionine, is biosynthesized in Escherichia coli as three isoforms (1). AK-I and AK-II each contain on the same polypeptide chain the third enzyme in the pathway homoserine dehydrogenase (HD-I and HD-II). AK-III occurs as a single activity. In all isoforms both AK and HD activities are activated by K$^+$ or NH$_4^+$ (2, 3). Cohen and colleagues (4) have studied the AK/HD isoforms extensively and provided unique insights to kinetic and regulatory mechanisms (12-14), as demonstrated for complex enzymes such as glutamine synthetase (15) and aspartate transcarbamylase (16, 17). A recently developed program, ISOBI, allows quantitative interpretation of isotope exchange data. Systematic simulation procedures can be used to derive a set of optimal individual rate constants for any Bi-Bi system, including those with multiple dead-end complexes (18).

Previous studies with AK-I showed that L-threonine is competitive versus both L-aspartate and ATP (19, 20), but with HD-I it acts as a partial but purely noncompetitive inhibitor versus L-homoserine (21, 22). Although substrates do not exhibit cooperative binding, L-threonine inhibits both activities of AK/HD-I in a sigmoidal manner (22, 23). In the allosteric (T-R) transition L-threonine favors the less active T-state, whereas L-aspartate and K$^+$ favor the more active R-state. L-Threonine antagonizes K$^+$ activation of AK-I, including conformational changes related to the T-R transition (6, 24, 25). A slow conformational change in AK-I is induced by L-threonine binding (26, 27), which buries previously exposed aromatic residues, exchangeable protons, and cysteine side chain groups (28-30), all related to hysteretic effects (31). In the absence of ligands, the conformation of AK/HD-I lies 90% toward the T-state (28). Both concerted (25, 28, 32) and sequential (27) allosteric mechanisms have been proposed for AK/HD-I. Based on transient kinetic (26, 27, 31), immuno-
logical (33), and spectroscopic (25, 34, 35) data, at least three distinctly different conformational states must be involved in allosteric regulation as follows.

$$\text{Thr} \quad R_c \Rightarrow R_c \Rightarrow T \quad \text{Asp} \quad K^+$$

EIEK methods are especially useful for elucidating exactly which kinetic steps were altered by bound modifiers, often modes that are nearly impossible to define by other approaches (36). The current investigation of E. coli HD-I is focused on determining the kinetic mechanism, along with optimal microscopic rate constants, as a means to then defining the mode of action of the allosteric modifier L-threonine.

**EXPERIMENTAL PROCEDURES**

**Materials**

The Tir-8 strain of E. coli, kindly supplied by Dr. G. N. Cohen, was used to prepare pure AK-HD-I by published methods (2, 37). All chemicals were ACS reagent grade, and biochemicals were of the highest purity obtainable from Sigma. L-ASA was synthesized by hydrolysis of N-acetyl-03-alanyl-L-lysine with hog kidney acylase, then ozonolysis of L-allylglycine (38). Stored in 4 N HCl at -20 °C, ASA was neutralized with 4 N KOH just before use and then kept on ice for up to 12 h. [14C]homoserine was from Amer sham Corp. [2,3-3H]NADP+ was synthesized from labeled NAD (50 µCi, 0.1 µmol, New England Nuclear-Du Pont) with NAD kinase (200 units, Sigma) at pH 7.5, 25 °C and was purified by ion exchange chromatography (see below). All aqueous solutions were prepared with distilled water that was freshly passed through a Barnstead Nanopure system.

**Initial Velocity Kinetics**

The rate of NADPH formation or loss was observed at 340 nm with a Perkin-Elmer Lambda 6B spectrophotometer as a function of the concentration of each substrate of the HD-I-catalyzed reaction. Reactions were thermostatted at 37 °C in 0.1 M CHES buffer, pH 9.0, containing 0.4 M KCl. $K_v$ values were calculated from $v$ versus [S] data by standard reciprocal replot methods using the ENZFITTER program (Elsevier-BIOSOFT).

**Fluorescence Titrations**

Enzyme (1.0 mg/ml) was thermostatted in pH 9.0 CHES/KCl buffer at 37 °C in a 0.6-ml quartz cuvette in a Perkin-Elmer model MPF-4B spectrophotometer. Upon excitation of Trp residues at 295 nm, emission of enzyme-bound NADPH was observed at 460 nm due to energy transfer to the cofactor, which avoided any correction for inner filter effects. Binding of NADP+ was observed by the quenching of Trp fluorescence emission at 340 nm. NADP or NAPD+ was added to enzyme-buffer solution over the ranges of 0-1.14 and 0-3.42 mM, respectively, which produced normal hyperbolic binding isotherms. Standard reciprocal replots of $\Delta F$, versus [cofactor] were used to calculate $K_v$ values. Addition of amino acids to enzyme plus nonreacting cofactor failed to elicit any significant fluorescence changes.

**Isope Exchanges**

Equilibrium isotope exchange experiments with HD-I were carried out according to established procedures (12, 13, 16). Reactions (0.2 ml) contained 100 mM CHES buffer, pH 9.0, plus 0.4 M KCl. Maximum concentrations of substrates were (mM): 5.0 NADPH, 12.5 NADP+, and 500 L-homoserine. Exchange rates were determined in triplicate. The concentrations of substrate pairs were varied from well below to well above their $K_v$ values as follows. Solution A (containing reaction components at twice their maximum concentrations) was mixed with solution B (lacking the varied components) in different proportions to the same final volume (0.1 ml). Water, enzyme, modifier, labeled substrate, or other components were then added to bring the final reaction volumes to 0.2 ml. After thermal equilibration for 5 min at 37 °C, 20 µg of AK-HD-I was added and the reactions incubated for 15 min to allow exact enzymic adjustment to equilibrium. Isotopic exchange was initiated by adding labeled substrate, [4H]NADP+ (0.02 µCi) or [14C]homoserine (0.1 µCi). To prevent perturbation of the equilibrium condition the micromoles of labeled material added was less than 1/100th that of the unlabeled pool. Exchange reactions were carried out for 10 min at 37 °C and then quenched.

Combinations of substrate pairs were also varied in the presence of 0.5 mM L-threonine, as done in kinetic mechanism studies. In addition, the modifier concentration was varied in the presence of substrates, held constant near or slightly above their half-saturation values (39).

**Quenching and Separations**

NADP+ and NAPD+ are unstable in solution outside the range of pH 7-9 (40) and were found to undergo breakdown upon refreezing, which made it impractical to quench exchange reactions by addition of acid/base or by freezing. NADP+ was NAPD+ exchange reactions were therefore quenched by addition of 4.8 ml ice-cold 20% ethanol/ water and kept on ice for up to 4 h before separation. NADP+ and NAPD+ were therefore separated in a Pharmacal Mono Q HR 5/5 anion exchange column, eluted in triethanolamine buffer by a KC1 gradient (41). To correct for cofactor instability, "zero time" control reactions were carried out in parallel with normal exchange reactions, quenching prior to addition of radiolabeled material.

He was ASA exchange reactions were quenched and the aldehyde group of ASA derivatized by addition of 0.1 M 2,4-dinitrophenylhydrazine (re-crystallized from ethanol) in 5 M HCl/0.6 M HCHO/ water. To ensure complete coupling, unlabeled carrier ASA was added to reactions containing low amounts of this component, and 2,4-dinitrophenylhydrazine was added at 20% excess over ASA. Quenched reactions could be flash-frozen in dry ice/ iPrOH and kept at -20 °C indefinitely. The homoserine and DNP-ASA fractions were separated on Whatman PK-5 (1000 pL) silica TLC plates by ascending elution with freshly prepared n-butanol/acetric acid/water (5/2/1). The DNP-ASA ($R_f$ = 0.65, yellow) and homoserine ($R_f$ = 0.37, ninhydrin positive) bands were removed from plates, powdered, packed into 2.5-ml plugged pipette tips, and eluted directly into scintillation vials with 2 x 2 ml 0.5 M KCl (for homoserine) or 2 x 1.5 ml 30% methanol/water (for DNP-ASA). Radioactivity was determined in Ecoscint mixture using an LKB model 1217 RackBeta liquid scintillation counter with external standard quench correction.

**Calculations**

For exchange of isotopic label at chemical equilibrium, $X' \equiv Y$, the micromoles of micromoles exchanged per min (R) was calculated according to the following.

$$R = -XY \ln(1 - F)/(X + Y)$$

where X and Y represent the micromoles of substrates present, t = time (min), and F is the fraction of isotopic equilibrium attained, $F = y/(x + y)$, where $x$ and $y$ are the disintegrations/min values observed in the respective pools (14).

**EIEK Data Analysis: Computer Simulation and Fitting**

EIEK methods allow simultaneous observation of rate-limiting and nonrate-limiting steps in both directions, including formation and breakdown of "inner" as well as "outer" complexes. The basis for distinguishing between sequential kinetic mechanisms from the shapes of saturation curves in EIEK experiments has been described in detail (12, 13), along with methods for defining effects caused by bound modifiers (39, 43) or site-specific mutations (18, 43).

**Kinetic Mechanism Studies**

The theory and practice of using the calculation program ISOBI to derive rate constants that optimally fit all available sets of EIEK data has been described elsewhere in detail (16, 18). Briefly, this involves the following procedures. (a) Enter the substrate concentrations and the exchanges observed in the actual laboratory experiments. Enter estimated initial values for each rate constant in the overall reaction, using (where possible) $k_{on}$ or $k_{off}$ values determined by rapid kinetic methods, assuming $k_{on}/k_{on} = K_v$ values determined by direct binding. If the observed exchange rates are unequal at saturation, covariant interconversion rates ($k, k'$) must be faster than substrate dissociation rates. At saturation, $k_{on}$ for substrates involved in the most rapid exchange are faster than $k_{on}$ for slower exchanging pairs. (b) Systematically vary the rate constants for structurally related substrate pairs (A/P, or B/Q), by trial-and-error, to produce an optimal fit to the experimental data. This yields a "best fit" set of explicit rate constants. (c) Validity checks: determine "confidence
limits,* the variation that causes less than 10–20% change in half-saturation or $R_{max}$ values for the simulated saturation curves, for each rate constant. Prove also that the optimal set of rate constants provides thermodynamic balance within all closed loops of the Bi-Bi scheme, and (using KINSIM [46]) predicts the correct initial velocities in the forward and reverse directions.

**Modifer Mode of Action (Data Analysis)**

**Phase I**—The first step in identifying which steps are altered involves using ISOBI to produce a set of model perturbations. The best fit set of rate constants (from kinetic mechanism studies) are used as a starting point. $K_a$ and $K_{on}$ for structurally related substrate pairs (e.g. A and P and B and Q) are systematically altered, and the resulting changes in $R_{max}$ and $K_c$ for each "experiment" are plotted as bar graphs. It is not unusual for each mode of modifier action to produce a set of bar graphs that is unique (42). Comparing model perturbation patterns to experimental ones most often serves to eliminate unlikely candidates, narrowing the focus of the search to the few most likely modes of action.

**Phase II**—Complexities in saturation patterns (e.g. inhibition effects) also usually necessitate a more detailed in-depth analysis. If modifier alters more than one set of constants or causes disproportionate changes in $K_a$ and $K_{on}$, identifying which rate constants the modifier alters may require systematic trial-and-error refitting of data. It then becomes necessary to use ISOBI to simulate the complete set of saturation curves for the most likely modes of action and then compare these curves in detail to those observed experimentally, including overall curve shapes and values for $R_{max}$ and $K_c$.

**RESULTS**

**Determination of $K_c$ at pH 9.0, 37 °C**

Data obtained with yeast enzyme (44) allow an estimate of $K_c = 900-1300$ for the HD-catalyzed reaction at pH 8. Taking $[H+]$ into account, one can calculate that $K_c = 90-130$ at pH 9. For EIEK studies with HD-I this magnitude of $K_c$ allows variation of all substrates in constant ratio near their $K_c$ values, and E. coli HD-I shows maximal activity near pH 8-9. For EIEK studies with HD-I this magnitude of $K_c$ was used in kinetic studies. $K_c$ values at pH 9.0, CHES buffer, 0.4 mM KCl, 37 °C, observing spectrophotometrically the extent of change in $A_{max}$, reactions initially containing a 3-fold excess of either ASA over NADPH or homoserine over NADP* were allowed to run to completion. In addition, isotopic transfer experiments, beginning with either a 2-fold excess of NADP* over [14C]-homoserine, or a 2-fold excess of L-homoserine over [3H]NADP*, were run to completion and the radiolabeled components chromatographically separated (see “Experimental Procedures”) and counted. Triplicate determinations produced an average value of $K_c = 100 (± 20)$.

**$K_c$ Values at pH 9.0, 37 °C**

Varying the concentration of each substrate in turn, using near-saturating concentrations of cosubstrate, initial velocity kinetics were carried out (see “Experimental Procedures”). The apparent $K_c$ values obtained for each substrate of HD-I are shown in Table I and are seen to be well within the range over which each substrate could be varied at equilibrium.

**Kinetic Mechanisms**

**Variation of All Substrates**—The first EIEK experiment usually performed during kinetic mechanism investigations involves varying all four substrates in constant ratio at equimolarities (9). These conditions minimize formation of dead-end complexes (45), which often complicate the interpretation of kinetic data with dehydrogenases (10–13). Saturation curves for the [14C]Hse ⇆ ASA and the [3H]NADP* ⇆ NADPH exchanges, varying of [all substrates], are shown in Fig. 1A.

These data reveal several important features of kinetic mechanism for HD-I. First, since ASA ⇆ Hse is faster than NADP* ⇆ NADPH, catalytic interconversion of central complexes (EAB EFPQ) cannot be rate-limiting. A kinetic "bottleneck" at catalysis would cause all exchange rates to become equal. Since the most rapid exchange must occur between those components released first or fastest from the central complexes, ASA and Hse dissociation must occur prior to (or faster than that for) cofactors. The sigmoidal shape of the NADP* ⇆ NADPH curve at $f = 0-0.2$ most likely results from multi-order kinetics at subsaturating substrate levels. Second, since ASA ⇆ Hse does not exhibit inhibition, amino acids cannot be released from the central complexes in a compulsory manner after cofactors. In contrast, inhibition of NADP* ⇆ NADPH above $f = 0.6-0.7$ indicates that either cofactors bind to enzyme prior to (or faster than) amino acids or dissociate after (or more slowly than) amino acids from the central complexes or both may be true. Alternatively, NADP* ⇆ NADPH inhibition could result from formation of a dead-end complex, but this is unlikely since abortive complex formation usually inhibits both exchanges in equal proportions.

**Variation of Substrate Pairs**—Structurally related substrate pairs (ASA/Hse or NADPH/NADP*) were varied in constant ratio, with cosubstrates held constant near half-saturation. Nonrelated pairs (NADP*/ASA or NADPH/Hse) were not varied, to avoid formation of dead-end complexes such as $E(NADP)(ASA)$ and $E(NADPH)(Hse)$. As shown in Fig. 1B, varying ASA and L-homoserine caused Hse ⇆ ASA to rise smoothly to a maximum, whereas NADP* ⇆ NADPH exhibited inhibition effects consistent with the preferred order random scheme hypothesized above. The rate of NADP* ⇆ NADPH equaled that for Hse ⇆ ASA at low homoserine and ASA levels, indicating that cofactor association–dissociation rates are as rapid as those for amino acids under these conditions. As saturation is approached, however, the faster dissociation of amino acids versus cofactors becomes dominant.

Upon varying NADPH and NADP* with fixed half-saturating levels of ASA and homoserine (Fig. 1C), both NADP* ⇆ NADPH and Hse ⇆ ASA exhibited weak partial inhibition effects. Because inhibition of Hse ⇆ ASA was not observed in Fig. 1A, the weak inhibition of both exchanges in Fig. 1C is very likely results from abortive complex formation. An alternative explanation is that, with high ASA and homoserine

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*Abortive complexes essentially remove active enzyme from solution; however, since they are derived from binary complexes (EA, EB, EP, or EQ), their formation can cause competitive inhibition effects versus the first substrate to bind but competitive effects versus the second substrate to bind to produce the central complex EAB or EQ. For example, formation of EAB from EA pulls the E-EA equilibrium toward EA, but Q competes with B for binding to EA via the equilibria EA-EAB versus EA-EQ.
but low NADP⁺ and NADPH, the branches of the Bi-Bi scheme involving the E(ASA) and E(Hse) binary complexes may operate at significant rates. Under saturating conditions, however, the faster dissociation of amino acids versus cofactors makes the alternative branches involving E-cofactor binary complexes dominant. Data-fitting and derivation of explicit rate constants with ISOBI (below) will be used to distinguish between these alternative possibilities.

Simulation and Fitting of EIEK Data with ISOBI—Applying the procedures and criteria outlined under "Experimental Procedures," ISOBI was used to fit the data for HD-I, using the substrate concentrations from the actual experiments in Fig. 1. An initial set of rate constants were chosen, assuming that catalysis was not rate-limiting, that association-dissociation rates for amino acids were faster than for cofactors and that $k_{off}/k_{on}$ approximated the $K_a$ or $K_m$ values in Table I (keeping in mind that very often $K_a$ does not equal $K_m$). By systematically changing these initial values, individually and in pairs, a set of rate constants were obtained by trial-and-error that provided optimal fit to the curves in Fig. 1. As depicted in Scheme I, these constants define a preferred order random kinetic mechanism. Confidence limits determined for each of these constants are also listed in Table II.

Saturation curves simulated with ISOBI, using the constants in Table II, are shown as the lines drawn in Fig. 1. These are seen to agree well with the experimental data in most cases in terms of both maximal exchange rates and curve shapes. The several curves that did not fit the experimental data well involved lower half-saturation values for the NADP⁺ ⇛ NADPH exchange, upon variation of $[A, B, P, Q]$ or $[A, P]$. Fig. 1, graphs A and C. The basis for these discrepancies are not yet well understood. It is hoped that more accurate determinations of some rate constants with wide confidence limits may improve the agreement.

The values shown in Scheme I have several distinctive features. First, on the reactant side, rate constants for formation of central complexes via the pathways $E-E(NADPH)$-$E(NADPH)(ASA)$ and $E-E(ASA)-E(NADPH)(ASA)$ are virtually equal, as are the dissociation rates for the binary complexes $E(ASA)$ and $E(NADPH)$. Faster dissociation of

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**Scheme I.** Preferred order random kinetic mechanism for E. coli homoserine dehydrogenase-I, with optimal values for rate constants (see text and Table II). Underlined values are sensitive to change, those in brackets are relatively insensitive.

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3 The near-identity of association rate constants for several substrates (Table II) is only apparent, since substrate concentrations also determine the actual rate, as in $k[S]$. Furthermore, due to wide confidence limits for certain constants, $k_{on}$ for $E-EA$ can be in the range of $1-5 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$, and for $E-EP$ and $EP-EPQ$, can be in the range of $4 \times 10^6$ to $1 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$. 
ASA versus NADPH from the central complex favors the pathway involving E(NADPH) over that involving E(ASA) by 3–5-fold. On the product side, the rate constants for association of homoserine and NADP+ to free E are also apparently nearly equal, but differences in dissociation rates favor loss of homoserine from the central complex prior to NADP+ by a factor of five. Second, certain constants have very narrow confidence limits, notably dissociation of ASA from E(NADP+)(ASA) and both on- and off-rates for E + Hse = E(Hse). Other constants were found to exhibit wide ranges of allowed variation, e.g. association-dissociation for the reactions E + NADP+ = E(NADP+)* and E(NADP+*) + Hse = E(NADP+)(Hse). Future efforts need to be directed toward determining these values more exactly by rapid kinetic methods. Third, the rate constants in Table II are validated by several criteria. Thermodynamic balance is achieved for both of the closed cycles in Scheme I, and calculations with KINSIM (46) gave forward and reverse initial velocities that agreed within 20% of those in Table I, assuming 5 mM cofactor and 50 mM L-ASA or 200 mM L-homoserine present initially.

Attempts to simulate the data in Fig. 1, assuming either a fully random or fully compulsory order scheme, were unsuccessful. Including various abortive complexes with either of these latter mechanisms (see below) invariably worsened the fit to the experimental data. In contrast, for the preferred order scheme, including an abortive complex improved the fit to the data.

Effects of Dead-end Complexes—For the preferred order random scheme, the effects of 12 possible dead-end complexes (16, 17) were modeled with ISOB, both individually and in combination. Virtually all of these caused Rmax to decrease for both exchanges (due to removal of active enzyme) and often altered S0.5 as well. It was unusual to observe either disproportional inhibition of one exchange versus another or inhibition only at high substrate levels. Binding of NADPH to the E(Hse) complex, however, disproportionately inhibited Hse ⇄ ASA upon varying the NADP+/NADPH pair and

**TABLE II**

<table>
<thead>
<tr>
<th>Process</th>
<th>$k_	ext{on}$</th>
<th>$k_	ext{off}$</th>
</tr>
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<tbody>
<tr>
<td>A. Substrate binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-EA</td>
<td>2.0 (±1.0) × 10^5</td>
<td></td>
</tr>
<tr>
<td>EB-EAB</td>
<td>6.0 (±2.0) × 10^6</td>
<td></td>
</tr>
<tr>
<td>E-EF</td>
<td>2.0 (±0.6) × 10^6</td>
<td>4.0 ± 10^2</td>
</tr>
<tr>
<td>EA-EAB</td>
<td>4.0 (±2.0) × 10^6</td>
<td></td>
</tr>
<tr>
<td>E-EP</td>
<td>(±1.0) × 10^6</td>
<td>(±6 × 10^7)</td>
</tr>
<tr>
<td>EQ-EPQ</td>
<td>2.0 (±0.6) × 10^6</td>
<td>4.0 ± 10^2</td>
</tr>
<tr>
<td>E-EQ</td>
<td>2.0 (±0.4) × 10^6</td>
<td>1.2 ± 10^2</td>
</tr>
<tr>
<td>EP-EPQ</td>
<td>(2.0 ± 10^8)</td>
<td>(2.0 ± 10^7)</td>
</tr>
<tr>
<td>B. Dead-end complexes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EQ-EAQ</td>
<td>2.0 (±0.5) × 10^6</td>
<td>6.0 ± 10^2</td>
</tr>
<tr>
<td>C. Catalysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EAB-EPQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. Hill numbers</td>
<td></td>
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</tr>
</tbody>
</table>

*Symbols for substrates: A, NADPH; B, ASA; P, NADP; Q, Hse.

If $K_d = k_{off}/k_{on}$, then using optimal values of each rate constants, with its confidence limits (Table II), one can calculate for the reactant side of the homoserine dehydrogenase reaction that $K_d \times K_d' = 3.6–144 \times 10^{-6}$, which clearly overlaps the range for $K_d \times K_d' = 0.9–8.9 \times 10^{-6}$. Similarly, on the product side, one can show that $K_p \times K_p' = 0.02–4.0 \times 10^{-6}$, which easily spans $K_p' \times K_p = 1.2–9 \times 10^{-4}$.

Improved the fit to the data in Fig. 1C. In fact, unless the E(Hse)(NADPH) complex was included, the kinetic constants that gave correct $R_{max}$ values for the preferred order random scheme failed to predict the correct initial velocities.

**Modifier Action: L-Threonine**

Saturation curves for the Hse ⇄ ASA and NADP+ ⇄ NADPH exchange rates as a function of [all substrates], in...
the presence and absence of 0.5 mM L-threonine, are shown in Fig. 2. The modifier caused several distinctive changes: stronger inhibition of the maximal rate for Hse = ASA than for NADP\(^+\) = NADPH and loss of the partial inhibition effects for NADP\(^+\) = NADPH above 0.5.

Upon variation of the Hse/ASA pair, holding NADP\(^+\) and NADPH constant, L-threonine inhibits primarily the maximal rate of Hse = ASA, but the distinctively sharp partial inhibition effect observed for NADP\(^+\) = NADPH with unmodified enzyme is essentially lost (Fig. 3). As observed in Fig. 2, L-threonine inhibits Hse = ASA more strongly than NADP\(^+\) = NADPH. The partial inhibition of NADP\(^+\) = NADPH observed for unmodified enzyme in Fig. 1B resulted from preferred order release of amino acids from the central complexes prior to cofactors. Intuitively, loss of this inhibition seems to indicate a change in kinetic mechanism, but such intuitive interpretations can be misleading (see below).

Upon variation of the NADP\(^+\)/NADPH pair, holding ASA and Hse constant (Fig. 4), L-threonine causes differentially stronger inhibition of Hse = ASA than of NADP\(^+\) = NADPH, plus loss of weak inhibition effects attributed to formation of the dead-end complex, E(Hse)(NADPH).

NADP\(^+\) = NADPH exchange showed little or no change in maximal rate, with some increase in the apparent \(K_a\) value for the saturation curve.

The effects of varying L-threonine with substrates held constant at partially saturating concentrations are shown in Fig. 5. As in Figs. 2–4, Hse = ASA is inhibited much more strongly than NADP\(^+\) = NADPH.

**Analysis of L-Threonine Perturbation Effects**

**Phase I**—For each experimental saturation curve in Figs. 2–4, the changes (perturbations) in \(R_{max}\) and \(K_a\) caused by L-threonine binding were calculated. Next, iSObi was used to model the data for threonine-modified HD-I, using rate constants for unmodified HD-I (Table II) as a starting point. On- and off-rates for structurally related substrate pairs were systematically increased or decreased. Increasing all possible combinations of rate constants failed to produce any perturbations that resembled those observed in the experimental data; therefore, these modes were not considered further. Decreasing certain rates did produce perturbations in \(R_{max}\) and \(K_a\) that resembled the experimentally observed effects of L-threonine. Specifically, these included decreases in either catalysis (\(k, k'\)) or in \(k_{off}\) and/or \(k_{on}\) for ASA and homoserine. Comparison of these perturbations as simple bar graphs failed to give a definitive match, however, due to complexities in curve shapes. This necessitated a more detailed modeling of the data.

**Phase II**—Modes of action identified in Phase I as most
likely candidates for L-threonine action were modeled as full saturation curves with ISOBI. Certain modes of action give curves that fit the experimental data in one or two but not in all experiments, making it important to compare complete sets of curves, presented in Fig. 6. First, decreasing \( k_{\text{off}} \) and \( k_{\text{on}} \) for Hse and ASA shifts the kinetic mechanism toward a random order scheme by decreasing differences in off-rates for amino acids vs. cofactors, and produced the curves shown in Fig. 6, labeled A and B, respectively. These modes of action were considered unlikely, however, since they fail to eliminate inhibition effects in NADP\(^+\) \( \rightleftharpoons \) NADPH upon varying [all substrates] or [Hse, ASA], even if the dead-end complex was weakened or deleted from the scheme.

Next, systematic trial-and-error refitting of the data for the Thr-modified enzyme was carried out, changing the kinetic mechanism from preferred order random to a fully random order scheme. Curves produced by these changes (Fig. 6, curves D) show a reasonably good match with those in Figs. 2–4. To optimally fit the data, however, it was necessary to increase the \( K_0 \) for the \( E(\text{Hse})(\text{NADPH}) \) abortive complex, which caused an increase in free enzyme and in both \( R_{\text{max}} \) values. To correct the \( R_{\text{max}} \) values, a proportional decrease in the entire set of rate constants was required. This in turn caused the KINSIM-calculated initial velocities to fall below the true experimental values by more than an order of magnitude. Failure to meet this essential criterion eliminated this complex mode of action from further consideration.

Finally, the effects of decreasing the minimal values for \( k \) and \( k' \) (Table II) by 10-fold are shown in Fig. 6, curves C. Considered as a complete set, these saturation curves agree with the experimental data to a greater extent overall than any other in Fig. 6. Agreement was better for the Hse \( \rightleftharpoons \) ASA exchange than for NADP\(^+\) \( \rightleftharpoons \) NADPH. The most significant deviations occurred for NADP\(^+\) \( \rightleftharpoons \) NADPH upon variation of [all substrates] and [Hse, ASA]. Furthermore, altering \( k \) and \( k' \) without changing any substrate on- or off-rates clearly maintains thermodynamic balance in all closed cycles, and KINSIM calculations showed that decreasing \( k \) and \( k' \) by 10-fold inhibits the initial velocity by 2/3, in excellent agreement with published results (22, 23). In conclusion, inhibition of catalysis (\( k, k' \)) is the most likely mode of action for L-threonine, since all other modes considered above fail in some way to meet the specified criteria. Although demanding, these procedures are essential for narrowing the field of candidate to the most likely modes of action. Detailed comparisons of multiple sets of curves is necessary for making the final discrimination of which mode provides the best fit.

**Modeling Inhibition of Catalysis**

To further validate the above conclusion regarding the mode of action of L-threonine on HD-I, the effects of inhibiting catalysis, without changing substrate on- or off-rates, were simulated with ISOBI. Fig. 7 demonstrates that as \( k \) and \( k' \) are progressively decreased the maximal rate of Hse \( \rightleftharpoons \) ASA is inhibited more strongly than NADP\(^+\) \( \rightleftharpoons \) NADPH, with little or no perturbation in apparent \( K_0 \) values for the saturation curve. The region of maximal rate for NADP\(^+\) \( \rightleftharpoons \) NADPH is broadened and shifted to higher substrate levels, which obscures the inhibition effects due to preferred order substrate binding. Without knowing the underlying cause, one might intuitively (but mistakenly) interpret this effect as a shift toward more random order substrate binding. In fact, the suppression of inhibition effects occurs without any changes in the relative rates for substrate association-dissociation. The changes in curve shape in Fig. 7 compare well with those in Fig. 2.

**DISCUSSION**

**Kinetic Mechanism**—The rate constants shown in Scheme I and Table II clearly demonstrate that the preferred order random kinetic mechanism for HD-I at pH 9, 30 °C, is determined by faster dissociation of amino acids than cofactors from the central complexes, not faster association of cofactors than amino acids to free enzyme. Rate limitation of initial velocity in either direction primarily depends upon the dis-
Action of L-Threonine—The present results illustrate the power and utility of simulation-enhanced EIEK methods for elucidating the mechanism of modifier action. The isotope exchange data for perturbation of HD-I by L-threonine are best explained by partial inhibition of catalysis, without any alteration in substrate association-dissociation rates, as depicted in Scheme II. Previous initial velocity data suggest that L-threonine acts as a pure noncompetitive partial inhibitor (21–23). Since the HD-I(L-threonine) complex retains approximately one-third of its original activity, the effector must bind to a site separate from those for substrates.

Interestingly, the simulations in Figs. 2–4 and 7 demonstrate that the “true” kinetic mechanism can be masked by making catalysis definitively rate-limiting. Regardless of the relative rates for substrate association and dissociation, the observed kinetic behavior becomes that of a random order system. An analogous effect was observed for E. coli aspartate transcarbamylase; a shift in temperature from 30 °C to below 15 °C caused a change from compulsory to apparent random order kinetic behavior, which was attributed to a dramatic change in the rate-limiting step (50).

If catalytic interconversion is not rate-limiting for unmodified enzyme, and L-threonine exerts its effect by acting on catalysis, what are the true values of $k$ and $k'$? Optimal fit to the EIEK data for unmodified enzyme only requires that $k \geq 2 \times 10^8$ s$^{-1}$ and $k' \geq 4 \times 10^7$ s$^{-1}$ (Table II). How much larger are $k$ and $k'$ than these lower limits? Since $K_i$ (L-threonine) \( \approx 0.2 \text{ mM} \) (1), for L-threonine binding $\Delta G = -RT\ln K_i \approx \pm 5.0 \text{ kcal/mol.}$ Each factor of 10 in rate or equilibrium requires \( \sim.4 \text{ kcal/mol.} \) Assuming 100% efficiency in transferring the energy of L-threonine binding to alter $k$ and $k'$, the maximum possible change in these rates would be 5/1.4 or 3.5 orders of magnitude. Assuming less than 100% efficiency of energy transfer, one can estimate that the actual values of $k$ and $k'$ probably no more than 10-fold above their lower limits, $2 \times 10^8$ s$^{-1}$ and $4 \times 10^7$ s$^{-1}$, respectively. This conclusion is in accord with the view that no evolutionary pressure would have existed to cause nonrate-limiting steps to become much faster than the rate-limiting steps.

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