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Biophysical Chemistry 64 (1997) 225–234

Biophysical Chemistry

Is substrate inhibition a consequence of allostery in aspartate transcarbamylase?

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Received 13 May 1996; revised 9 July 1996; accepted 10 July 1996

Abstract

Aspartate transcarbamylase (ATCase) is a highly regulated, multisubunit enzyme that catalyzes the first regulated step in pyrimidine biosynthesis. Although ATCase exhibits strong substrate inhibition (the reduction of enzyme activity at high substrate concentrations), the mechanism of substrate inhibition has not been investigated. At the molecular level, substrate inhibition may result either from local events at the active site or from global or specific long-range allosteric effects. We have compared the results of fitting kinetic data to several models: (a) a semi-empirical steady-state kinetic model that includes cooperative substrate binding (described by a Hill coefficient) and partial uncompetitive substrate inhibition, (b) a nested allosteric model developed to analyze substrate inhibition of the ATPase activity of GroEL, an enzyme with a quaternary structure analogous to ATCase (O. Yifrach and A. Horovitz, Biochemistry, 34 (1995) 5303), and (c) purely concerted models, including a model originally proposed by Monod et al. (J. Monod, J. Wyman and J.P. Changeux, J. Mol. Biol., 12 (1965) 88). Model (a) is the first kinetic equation for ATCase that both fits the data and returns physically realistic values for all parameters, but it is a modified Hill equation and thus returns little or no molecular mechanistic information. The nested allosteric model (b), which assumes concerted cooperativity within each catalytic trimer of ATCase and sequential cooperativity between trimers, is unlikely to be the correct model for ATCase, since isolated catalytic trimers, which cannot exhibit the sequential cooperativity of the model, still exhibit substrate inhibition. Analysis of concerted models (c) shows that a two-state model is inadequate to account for substrate inhibition in ATCase. Further, although unique fits to a three-state model cannot be obtained, because the parameters are highly correlated, several sets of parameter values fit the data well and are in accord with other experimental results. These results indicate that substrate inhibition in ATCase may be the consequence of allostery, and that further experimental investigation is warranted.

Keywords: Cooperativity; Modeling; Nonlinear analysis; Data simulation; Enzyme kinetics; GroEL

1. Introduction

Aspartate transcarbamylase (ATCase) from *Escherichia coli* is an extensively studied, highly regulated enzyme. ATCase catalyzes the condensation of aspartate and carbamyl phosphate to form carbamyl aspartic acid in the first committed step of pyrimidine biosynthesis. While it binds carbamyl phosphate non-cooperatively, it binds aspartate with positive homotropic cooperativity, is positively heterotropically regulated by ATP, is negatively heterotropically regulated by CTP, binds CTP with negative cooperativity, exhibits synergistic enhancement of

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PII S0301-4622(96)02204-1

CTP's inhibitory effect in the presence of UTP, and has a Bohr effect (see [1–7] for recent reviews).

Substrate inhibition in ATCase has been recognized for decades [8], but its mechanism remains unknown. However, it is an important issue, since in ATCase and other systems it occurs at sufficiently low substrate concentrations that ignoring it and truncating data can result in substantial errors in the values derived for critical parameters such as the apparent binding constant, maximal velocity and Hill coefficient [9,10]. Substrate inhibition may also be significant physiologically, although addressing this issue with certainty requires accurate knowledge of the concentrations of highly localized intracellular pools. Finally, the possibility that substrate inhibition in ATCase has a homotropic allosteric basis, as has been proposed in other systems (see [11,12]), warrants investigation, since it would have important implications in terms of understanding the allosteric repertoire of this important model system.

We have carried out kinetic titrations of ATCase, its catalytic trimer, and human ornithine transcarbamylase (OTCase), which is homologous to the catalytic trimer of ATCase, under conditions that allow substrate inhibition to be analyzed and have compared the results of fitting these data to several models. We describe a semi-empirical steady-state rate equation based on the Hill equation that fits kinctic titration curves for ATCase and returns physically realistic values of the parameters. This equation is similar to an earlier, widely used equation which fits the data but has highly correlated parameters and fails to return realistic values for all parameters except the Hill coefficient [9]. We next tested a nested allosteric model developed for the GroEL chaperonin [11], whose quaternary structure is analogous to that of ATCase. We also investigated several purely concerted models, including one originally described by Monod et al. [13]. Relevant experimental results are assessed and further experimental studies to discriminate among these models are proposed.

2. Methods

Plasmids for expression of *E. coli* ATCase and human OTCase were provided by Dr E.R. Kantrowitz (Department of Chemistry, Boston College) and Dr Mendel Tuchman (Department of Pediatrics, University of Minnesota), respectively. ATCase and OTCase were purified as described elsewhere [14,15]. Catalytic trimers were prepared following Yang et al. [16] with modifications introduced by Burz and Allewell [17]. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

ATCase, OTCase, and the ATCase catalytic trimer were assayed using the colorimetric assay developed by Prescott and Jones [18] as modified by Pastra-Landis et al. [9], with further minor modifications as described by Yuan et al. [19]. ATCase and its catalytic trimer were assayed at a concentration of 0.1 μg ml^{-1} in buffer containing 20 mM Tris–HCl (tris(hydroxymethyl)aminomethane hydrochloride), 20 mM bis–Tris (bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane), 20 mM Caps (3-cyclohexylamino-1-propanesulfonic acid), 0.2 mM dithiothreitol, 0.2 mM EDTA (ethylenediaminetetraacetic acid), pH 8.3 [9,19]. OTCase was assayed in 50 mM Tris–acetate (tris(hydroxymethyl)aminomethane acetate), pH 8.3, at a concentration of 0.2 μg ml^{-1}. All aspartate titrations were performed with saturating carbamyl phosphate (4.8 mM; ~ 3 orders of magnitude above the Kd).

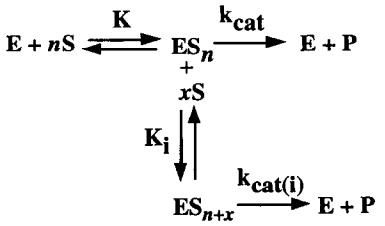
Nonlinear regression was performed using the nonlinear least-squares program NONLIN [20] on an IBM RS/6000 Model 550. Fits were performed using multiple initial guesses for the parameters to ensure finding the global variance minimum. Simulations were carried out with both NONLIN and KALEIDAGRAPH (Abelbeck Software).

3. Results

3.1. A semi-empirical kinetic model

Previous analyses of substrate inhibition in ATCase have modeled it as either uncompetitive [8] or partially uncompetitive [9]. In both cases, substrate binds as an inhibitor to a species to which substrate is already bound productively. In completely uncompetitive inhibition, the inhibited complex is unable to convert substrate to product, while in partially uncompetitive inhibition, the rate at which substrate is converted to product is simply reduced. Since the inhibition of ATCase does not approach zero even at very high substrate concentrations, the inhibition appears to be only partial. Incorporating a Hill coeffi-

cient description of cooperativity (see [22]) into a standard description of partial uncompetitive inhibition (compare [21]) yields the general reaction scheme:



The steady-state rate equation for this reaction is a modified Hill equation with additional terms in both the numerator and denominator for substrate inhibition:

$$\text{Velocity} = \frac{V_{max} + V_i\left([S]^x/K_i^x\right)}{1 + \left(K^{n_H}/[S]^{n_H}\right) + \left([S]^x/K_i^x\right)} \quad (1)$$

V_{max} and V_i correspond to the catalytic constants k_{cat} and $k_{cat(i)}$, respectively, and n_H is the Hill coefficient. The exponent x is a second Hill coefficient that allows for the possibility that binding of substrate in the inhibitory mode may also be cooperative. This is a more general form of the kinetic model introduced by Pastra-Landis et al. [9]. To obtain convergence, the value of x must be fixed. A value of $x = 1$, used in previous analyses, is known to yield physically unrealistic values for all kinetic parameters except the Hill coefficient [9]. The integer value of x that gives the best fit (lowest variance) for Eq. (1) is $x = 2$, although the values of the other five parameters obtained with values of x between 1.5 and 3 are within the confidence intervals of the fits with $x = 2$ reported in Table 1. Fitted values for the other parameters rapidly become physically unrealistic below $x = 1.5$. For simplicity, we used $x = 2$ in all fits reported here.

Figs. 1 and 2 show two extremes in the magnitude of aspartate inhibition in ATCase as a function of temperature. Titrations are carried out at saturating concentrations of carbamyl phosphate. The magni-

Table 1

	ATCase, 31°C	ATCase, 37°C	c_3 trimer, ATCase	OTCase
A. Fits to the partial uncompetitive inhibition Hill equation [a]				
V_{max}	42.6 (38.5, 47.0)	41.3 (38.0, 44.9)	17.3 (15.8, 18.9)	3.84 (3.41, 4.36)
V_i	13.9 (13.3,14.5)	23.1 (22.3, 23.8)	11.1 (10.8, 11.4)	1.87 (1.70, 2.03)
K	19.8 (18.2, 21.6)	22.6 (20.8, 24.6)	10.4 (8.7, 12.3)	0.287 (0.236, 0.348)
n_H	2.6 (2.4, 2.8)	2.3 (2.1, 2.5)	1.23 (1.09, 1.35)	1.61 (1.25, 1.91)
K_i	53.6 (46.1, 60.5)	75.2 (60.3, 89.1)	81.8 (60.1, 101.1)	3.31 (2.34, 4.12)
σ [b]	0.294	0.291	3.8e − 2	3.6e − 2
B. Fits to the Hill equation [a,c]				
V_{max}	31.7 (29.8, 34.0)	33.9 (32.6, 35.5)	15.2 (14.2, 16.6)	3.91 (3.46, 4.54)
K	16.0 (15.0, 17.2)	18.8 (18.0, 19.8)	8.2 (7.0, 9.8)	0.298 (0.240, 0.378)
n_H	3.1 (2.5, 3.6)	2.8 (2.4, 3.2)	1.35 (1.1, 1.6)	1.5 (1.1, 1.8)
σ [b]	1.17	1.02	0.181	4.5e − 2
C. Fits to the nested allosteric model [a]				
$V_{max(1)}$	69.4 (67.8, 71.0)	77.1 (75.0, 79.4)	33.95 (32.6, 36.3)	7.45 (7.14, 7.94)
$V_{max(2)}$	15.0 (14.4, 15.6)	24.3 (23.5, 24.9)	11.6 (11.2, 11.9)	1.93 (1.74, 2.11)
K_R	0.53 (0.45, 0.67)	3.45 (2.9, 4.3)	7.70 (6.46, 9.94)	0.125 (9.94e − 2, 0.179)
L_1	2.9e − 5	3.9e − 3	0.653 (0.354, 1.19)	8.5e − 2
	(1.6e − 5, 5.1e − 5)	(2.3e − 3, 6.3 e − 3)		(3.8e − 2, 0.18)
L_2	4.3e − 7	4.8e − 5	3.3e − 4	3.1e − 5
	(2.4e − 7, 7.7e − 7)	(2.3e − 5, 8.8e − 5)	(1.2e − 4, 7.2e − 4)	(8.6e − 6, 7.6e − 5)
σ [b]	0.576	0.421	7.2e − 2	4.3e − 2

[a] All velocity parameters (V_{max}, V_i, $V_{max(1)}$, and $V_{max(2)}$) are in mmol mg^{-1} h^{-1}. All dissociation constants (K, K_i, K_R) are in mM aspartate for the ATCase holoenzyme and catalytic trimer, and in mM ornithine for OTCase. Values in parentheses are the 65% confidence intervals of the fitted parameters.

[b] The variance of the fit $\sigma = \Sigma(Y_i - Y_{calc})^2$/degrees of freedom.

[c] Fits to the standard Hill equation were performed on the truncated data sets shown in the B panels of Fig. 1Fig. 2Fig. 3Fig. 4.

tude of substrate inhibition at 25°C (data not shown, see also [9,19]) is comparable to that shown at 31°C (Fig. 1). Other solution variables, for example pH and ionic strength, and some single site mutations also have dramatic effects on the magnitude of substrate inhibition (data not shown, see also [9,19]). The large variation in the magnitude of substrate inhibition with modest changes in conditions argues against it being an artifact of the assay or a result of ionic strength or non-ideality effects. Further, out-of-order substrate binding is unlikely to be a significant factor since (a) the reaction mechanism for ATCase is preferred order (i.e. not obligate order) with aspartate binding second (reviewed in [2,3,5,6]), (b) titration with either substrate exhibits substrate inhibition [8], (c) increasing the concentration of carbamyl phosphate does not alleviate aspartate inhibition (data not shown), and (d) aspartate inhibition never approaches 100% (e.g. see Figs. 1–4).

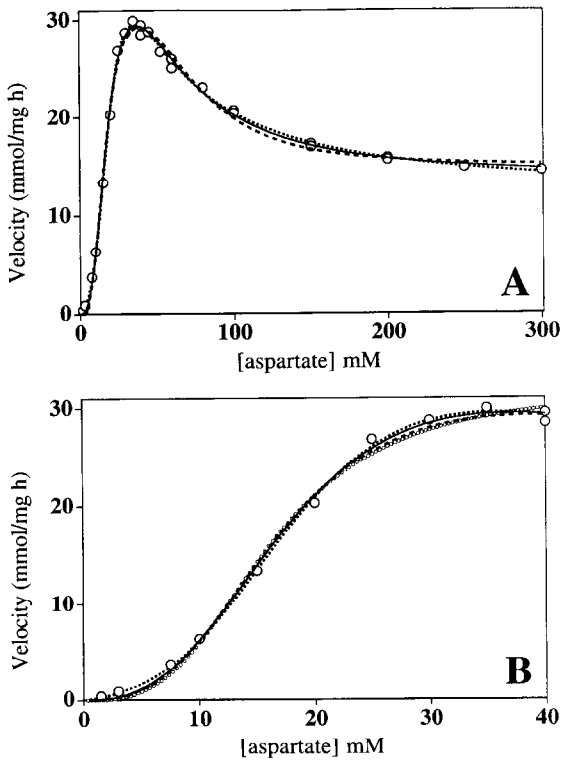


Fig. 1. Aspartate titration data for the ATCase holoenzyme at 31°C. Best-fits of Eqs. (1) and (2) are shown as solid and dashed lines, respectively. The dotted line is simulated using Eq. (4). Panel B shows a magnification of the data at the lower substrate concentrations, the beaded line shows the best fit to the standard Hill equation.

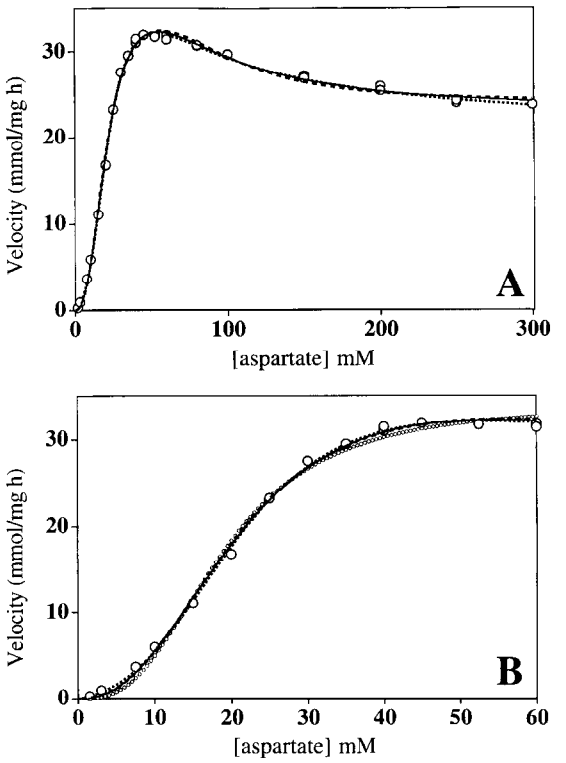


Fig. 2. Aspartate titration data for the ATCase holoenzyme at 37°C. Best-fits of Eqs. (1) and (2) are shown as solid and dashed lines, respectively. The dotted line is simulated using Eq. (4). Panel B shows a magnification of the data at the lower substrate concentrations, the beaded line shows the best fit to the standard Hill equation.

The solid lines in Figs. 1 and 2 are the fits to Eq. (1). Values of the parameters are given in Table 1. In all cases, the fitted parameters for this model closely match the values that would be derived by visual inspection. K and K_i are comparable to the $[S]_{1/2}$ values for the ascending and descending arms of the curve, respectively. The fitted V_{max} is expected to be larger than the maximum because the observed velocity is the sum of increasing and decreasing components. The earlier the onset of substrate inhibition (i.e. the higher the K/K_i ratio) the greater the discrepancy should be, as is the case. The fitted Hill coefficients are also reasonable, and agree well with those obtained from Hill plots (data not shown).

These checks are important both because the kinetic parameters for the ATCase system are still often determined simply by visual inspection of data in the range shown in the B panels of Figs. 1–4, and because they indicate that the fitted values of these parameters are physically realistic. The beaded lines in the B panels of the figures show the fits of that portion of the data to the standard Hill equation. Parameter values from these fits are given in Table 1 and illustrate the point made previously [9,10], that truncation of the data can alter the determined values since the contribution of the inhibition to the overall curve shape cannot be assessed.

Although Eq. (1) is largely phenomenological, it is the first to provide unique, physically reasonable fits to full titration data for ATCase. It was required to analyze quantitatively the pH dependence of the catalytic activity of ATCase and mutants of ATCase [19]. It enables a semi-subjective approach to be replaced with a more objective approach. Since an isotherm with 15–20 points is easily and uniquely fit, it does not require any increase in the amount of data collected, but simply requires that they be collected to concentrations where substrate inhibition plateaus.

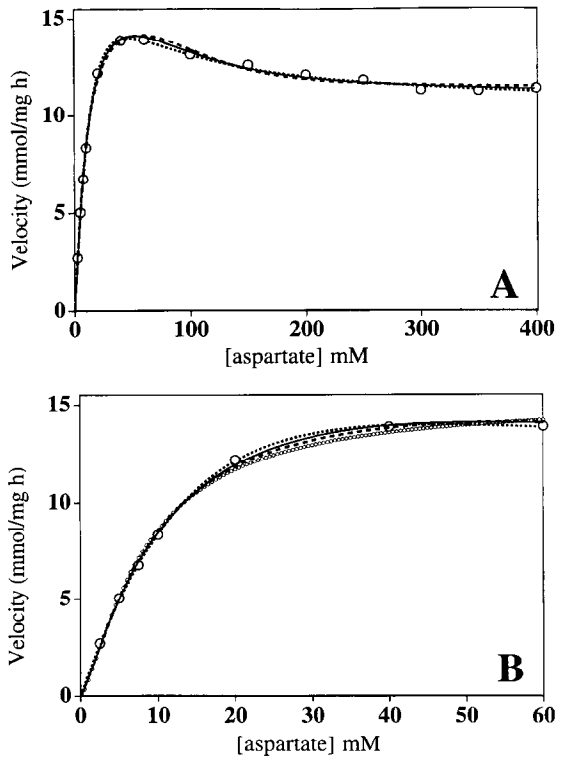


Fig. 3. Aspartate titration data for the catalytic trimer of ATCase. Best-fits of Eqs. (1) and (2) are shown as solid and dashed lines, respectively. The dotted line is simulated using Eq. (3). Panel B shows a magnification of the data at the lower substrate concentrations, the beaded line shows the best fit to the standard Hill equation.

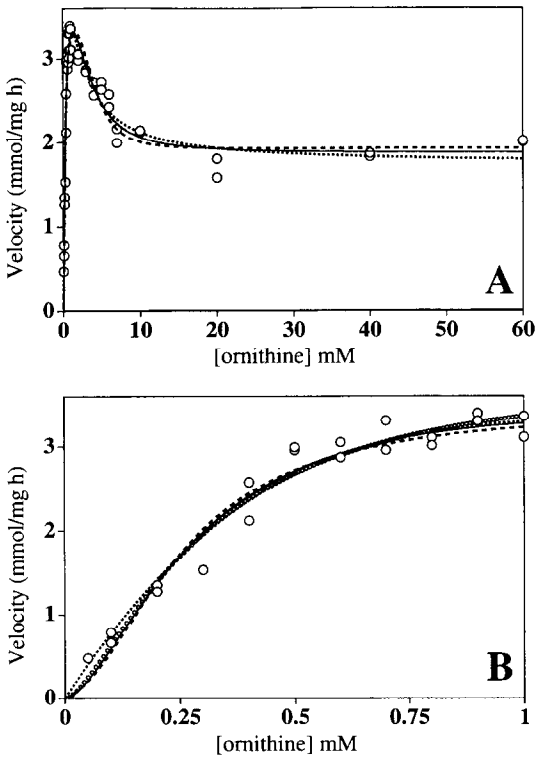


Fig. 4. Ornithine titration data for human ornithine transcarbamylase (OTCase). Best-fits of Eqs. (1) and (2) are shown as solid and dashed lines, respectively. The dotted line is simulated using Eq. (3). Panel B shows a magnification of the data at the lower substrate concentrations, the beaded line shows the best fit to the standard Hill equation.

3.2. A nested allosteric model

Like ATCase, the ATPase activity in the GroEL chaperonin is subject to substrate inhibition [11] and the shapes of the titration curves are strikingly similar to those shown in Fig. 1 for ATCase. ATCase and GroEL also share a common structural feature, a pair of stacked rings. In ATCase, the two catalytic

trimers are stacked, while GroEL consists of two stacked seven-membered rings. Yifrach and Horovitz [11] show that the ATPase kinetics of GroEL (including substrate inhibition) are fit by a model that nests an MWC model [13] for positive cooperativity in each ring within a KNF model [23] for apparent negative kinetic cooperativity between the two rings. Allosteric constants (L_1 and L_2) describe the two conformational transitions within the oligomer:

$$TT \overset{L_1}{\leftrightarrow} TR(RT) \overset{L_2}{\leftrightarrow} RR$$

The rate equation for this nested allosteric model is [11]:

$$\text{Velocity} = \frac{0.5V_{max(1)}L_1([S]/K_R)(1+[S]/K_R)^{N-1} + V_{max(2)}L_1L_2([S]/K_R)(1+[S]/K_R)^{2N-1}}{1+L_1(1+[S]/K_R)^N + L_1L_2(1+[S]/K_R)^{2N}} \quad (2)$$

where $V_{max(1)}$ and $V_{max(2)}$ are the maximal velocities of the TR and RR states, respectively, N is the number of sites within a single ring (the MWC cooperative unit, e.g. $N = 7$ for GroEL) and K_R is the dissociation constant of substrate from the R subunits. The allosteric constants L_1 and L_2 are defined as [TR]/[TT] and [RR]/[TR], respectively, in the absence of substrate. The model assumes that the T subunits do not bind ligand and that fractional saturation is directly proportional to V/V_{max}.

The functional and structural similarity between GroEL and ATCase suggested to us that this model might be applicable to ATCase, with the catalytic trimer as the MWC cooperative unit ($N = 3$ in Eq. (2)). The dashed lines in Figs. 1 and 2 show the fit of the nested allosteric model to the data for ATCase; parameter values are given in Table 1. The variance of the fits and the span and lack of systematic patterns in the residual plots (not shown) indicate that the nested allosteric model fits the data well. The fractional change in the variance allowed in fitting had to be set somewhat lower than with Eq. (1) to achieve smooth convergence to the global minimum from different initial guesses.

Since the substrate inhibition depends upon interactions between rings in the nested allosteric model, the model predicts that separating the rings will eliminate substrate inhibition. This prediction can be tested with ATCase, since isolated catalytic trimers retain catalytic activity. As shown in Fig. 3, they also retain substrate inhibition. Although the onset of weak substrate inhibition in the catalytic trimer has been observed under other conditions [8,9], Fig. 3 shows the full titration of the trimer to an inhibition plateau. Self-association cannot explain the effect, since the isolated trimers do not associate even at protein concentrations five orders of magnitude higher than used in this experiment [24]. Nevertheless, the data for the catalytic trimer can be fit to the nested allosteric model with any value of N between 1 and 3 (Fig. 3 and Table 1).

Since substrate inhibition of the catalytic trimers of ATCase is reduced relative to the holoenzyme, the possibility exists that substrate inhibition has more than one mechanistic origin and that interaction between the rings in the holoenzyme is required to achieve the maximal effect. However the results shown for human OTCase shown in Fig. 4 suggest that this is unlikely to be the case. Human OTCase is functionally and structurally homologous to the ATCase catalytic trimer [25]. It catalyzes the transcarbamylation of ornithine rather than aspartate, shares 46% sequence similarity with the catalytic trimer of *E. coli* ATCase, and exists as a trimer that does not self-associate under our assay conditions [15]. Porcine OTCase, which is 95% sequence homologous to human, shows no self-association even at concentrations four orders of magnitude higher than our assay conditions [26]. As shown in Fig. 4, however, human OTCase exhibits pronounced substrate inhibition, comparable in magnitude to that of ATCase holoenzyme at 31°C, under the conditions used. The nested allosteric model fits the data well, demonstrating again that a model that fits the data well need not be correct.

3.3. Fully concerted models

Although the nested allosteric model is not physically realistic, the fact that it fit the data well suggested that other allosteric models were worth investigating. Monod et al. [13] describe a kinetic exten-

sion of their two-state binding model that could account for substrate inhibition, in which the higher affinity state (R) is catalytically inactive. The equation for a trimer is:

$$\text{Velocity} = \frac{V_{max} L([S]/K_T)(1 + [S]/K_T)^2}{L(1 + [S]/K_T)^3 + (1 + [S]/K_R)^3} \quad (3)$$

Here, K_T and K_R are substrate dissociation constants for the T and R states, respectively. L is the $T \rightarrow R$ allosteric constant and corresponds to the descending portion of the titration curve. Monod et al. [13] did not examine the applicability of this model to any specific system, but it has been proposed to account for substrate inhibition in insect actomyosin [12]. This model can describe the data for both the ATCase catalytic trimer and OTCase (see below).

A more complex model that includes cooperativity of both productive and inhibitory substrate binding is needed to describe the behavior of the ATCase holoenzyme. This can be accomplished with a three-state concerted model:

$$T \overset{1/L}{\leftrightarrow} R \overset{M}{\leftrightarrow} S$$

where L has its original definition of [T]/[R] [13], and M is defined as [S]/[R] (and thus will have values < 1). The special case of this general model in which the T-state is catalytically inactive is the simplest concerted model that accounts for both cooperative productive substrate binding and substrate inhibition:

$$\text{Velocity} = \frac{V_{max(R)}([S]/K_R)(1 + [S]/K_R)^5 + V_{max(S)} M([S]/K_S)(1 + [S]/K_S)^5}{L(1 + [S]/K_T)^6 + (1 + [S]/K_R)^6 + M(1 + [S]/K_S)^6} \quad (4)$$

where K_T, K_R, and K_S are the aspartate dissociation constants for the T, R, and S states. This kinetic equation is based on the three-state binding equation of Minton and Imai [27]. Both Eqs. (3) and (4) (like Eq. (2)) assume that fractional saturation equals V/V_{max}, and that V_{max} for each state equals the product of k_{cat} for that state and the total enzyme concentration. The most general form of the three-state model requires only the addition of a term for catalysis in the T-state ($V_{max(T)}([S]/K_T)(1 + [S]/K_T)^5$) to the numerator of Eq. (4).

In contrast to the models discussed in Sections 3.1 and 3.2, unique fits cannot be obtained with either Eq. (3) or Eq. (4). Convergence can be obtained to parameter sets that fit the data, but a global variance minimum cannot be determined due to very high correlations among the parameters. Simulation studies suggest that this is an intrinsic feature of the combination of these functions and the curve shape generated by substrate inhibition, and not a function of data set size, range, error, or spacing, since very large sets of error free data simulated with Eqs. (3) and (4) cannot be uniquely fit back to Eqs. (3) and (4). Reparameterizations of Eqs. (3) and (4) did not alleviate this problem, although all possibilities were not exhausted. The parameters M and K_T are the most highly correlated with the other parameters and their estimation is the most uncertain as they cannot be properly minimized. If initial guesses for M and K_T are within about an order of magnitude of the values shown in Table 2, the other parameters consistently converge to similar but not identical values. This was the only physically meaningful minimal region of the parameter space identified. This failure to distinguish a unique global minimum does not invalidate the model, since a model is invalidated only when no set of parameters can be found that fits the data, as discussed in [20,28–30]. For example, this criterion was used to eliminate all special cases of the general three-state model simpler than Eq. (4) for the ATCase holoenzyme, and to determine that Eq. (4) is not applicable to GroEL (data not shown). In contrast, curves can be simulated with reasonable values of the parameters that fit all the experimental data. The application of Eq. (3) to the ATPase activity of insect actomyosin was also accomplished by data simulation [12]. The dotted lines in Figs. 1–4 show the curves simulated with the parameter values

Table 2
Parameter values for simulations with two- and three-state concerted models [a]

a. Two-state concerted model, Eq. (3)		
	c_3 trimer, ATCase	OTCase
V_{max}	42.6	15.4
L	248.7	61.6
K_T	36.9	1.8
K_R	4.01	0.23
σ [b]	1.1e−2	3.0e−2
b. Three-state concerted model, Eq. (4)		
	ATCase, 31°C	ATCase, 37°C
$V_{max(R)}$	73.4	65.5
$V_{max(S)}$	2.08	9.08
L	22.6	13.7
M	3.0e−6	4.8e−6
K_T	5.0e8	6.0e8
K_R	23.0	27.8
K_S	2.02	2.87
σ [b]	0.16	0.15

[a] All velocity paramters (V_{max}, $V_{max(R)}$, and $V_{max(S)}$) are in mmol mg^{-1} h^{-1}. All dissociation constants (K_R, K_T, and K_S) are in mM aspartate for the ATCase holoenzyme and catalytic trimer, and in mM ornithine for OTCase.
[b] The variance of the fit $\sigma = \Sigma(Y_i - Y_{calc})^2$/degrees of freedom.

of one of the variance minima of Eq. (3) for the catalytic trimer of ATCase and for OTCase, and of Eq. (4) for the ATCase holoenzyme; the values are given in Table 2.

4. Discussion

The models examined in this study have many similar features yet differ markedly from one another in character. The allosteric models represent two possible molecular models of the phenomenological partial uncompetitive kinetic model. Since all three fit the data, the question of which one(s) are applicable and warrant further investigation must be decided on other grounds. All three models postulate that saturating the enzyme with substrate reduces its catalytic efficiency. At the molecular level this could be accomplished either by heterotropic allostery (binding of substrate at an inhibitory second site) or by homotropic allostery (inhibition as a consequence of a conformational change accompanying binding at the active site). Both allosteric models are homotropic models of the K–V type, in which the conformational states differ in both affinity and catalytic efficiency [13].

The nested allosteric model can be ruled out for ATCase on at least three counts. (It is worth noting that to our knowledge similar inquiries have not been performed for GroEL.) Most tellingly, it does not predict that substrate inhibition will persist in isolated catalytic trimers. It also does not predict previous results obtained for hybrid holoenzymes containing two active c chains and four inactive chains [31]. Schachman and associates [31] showed that the kinetics of molecules with both active chains in one catalytic trimer are identical to the kinetics of molecules with one active chain in each trimer. These experiments indicate that the asymmetry predicted by the nested allosteric model that results from the two rings switching conformation independently is not a feature of the ATCase system. Third, the fitted values of the allosteric constants L_1 and L_2 for the holoenzyme at 31°C correspond to free energy changes ($\Delta G = -RT \ln L_n$) of 6.3 kcal mol^{-1} for the TT → TR transition and 8.9 kcal mol^{-1} for the TR → RR transition. These values are much larger than any estimates for the T → R transition derived by a wide variety of approaches, both model dependent and model independent. Previous values range from 0.5–3.3 kcal mol^{-1} (reviewed in [7]).

The possibility that there might be heterotropic inhibitory aspartate binding sites was first suggested by Heyde [32], who postulated a second binding site for dicarboxylic acids near the active site to reconcile conflicting models of the kinetic mechanism of ATCase. Such a site would be difficult to detect by binding studies in solution, because of the high ligand concentrations required, but it is notable that no stoichiometric titration of ATCase or its catalytic trimer with any ligand or ligand analog has yet yielded a stoichiometry greater than the number of active sites (reviewed in [7]). Binding of succinate and malonate at sites other than the active site has not been detected crystallographically in experiments carried out at saturating ligand concentrations (reviewed in [4–6]), although such sites might be undetectable or overlooked if the ligand were bound loosely. The bisubstrate analog PALA (*N*-phosphonacetyl-L-aspartate), which binds much more

tightly, clearly does not have additional sites in solution or in the crystal (reviewed in [4–6]), but it has an additional phosphonacetyl group. Hence the available evidence suggests that there is no discrete inhibitory site, although the possibility cannot be ruled out absolutely.

Even in the absence of unique fits, the two- and three-state concerted models yield some intriguing implications and correlations with other studies of ATCase.

(a) The two-state model (Eq. (3)) requires that the catalytic trimer of ATCase, like the holoenzyme, undergoes a conformational change upon substrate binding, as has been observed [33,34].

(b) Accounting for substrate inhibition in the holoenzyme appears to require a minimal three-state allosteric model. Even a highly inclusive two-substrate extension of the two-state model [35] cannot account for the holoenzyme kinetics (data not shown). No second conformational change has been reported for the holoenzyme, but few experiments have been carried out in the appropriate concentration range.

(c) The concerted models postulate positive cooperativity of binding, in which affinity increases with degree of saturation, and attribute substrate inhibition to negative regulation of the catalytic rate. Hence, direct binding studies are expected to show positive cooperativity. This is certainly the case for the ATCase holoenzyme (reviewed in [7]). For the catalytic trimer, the two-state model of Eq. (3) permits non-cooperative binding to the catalytically productive state across a wide substrate concentration range prior to the cooperative switch to the inhibited state, which is consistent with the non-cooperativity of most direct binding and enzyme kinetic titrations of the catalytic trimer (reviewed in [7]); nonetheless, cooperative binding of PALA to the catalytic trimer has been observed [36].

(d) The simplest three-state concerted model that fits the data for the holoenzyme postulates that the low-affinity T-state is catalytically inactive and returns an extremely large value of K_T. This is consistent with a number of experimental results for the holoenzyme that suggest that the binding affinity of the T-state for aspartate and its catalytic efficiency are extremely low or negligible [37–40].

(e) The free energies corresponding to the allosteric constant L for the holoenzyme (1.9 and 1.6 kcal mol^{-1} at 31 and 37°C, respectively) correspond well to previous estimates (reviewed in [7]).

Substrate inhibition is a phenomenon that requires more attention than it has received in the past, particularly since the results presented here indicate that it may be part of the allosteric repertoire of ATCase. Since three states appear to be required to fit the kinetic data for the holoenzyme, the question of whether a third state exists is critical. The occurrence of intermediate states has been proposed, both by crystallography and in solution; however, their existence remains controversial [1], and no unified understanding of their structural, binding and catalytic properties has emerged (reviewed in [5,6]). It is important to note that the structural changes of various states need not parallel the observed non-monotonic kinetic properties; for example, the R-state in Eq. (4), which has a higher catalytic efficiency than either the T- or S-states, might have an intermediate structure. If a third conformation exists, it may involve only minor structural changes, making its detection difficult. Resolving the existence of a third allosteric state in the hemoglobin system was a difficult and complex task [41]. Furthermore, such a putative third state might correspond to either R or S in Eq. (4), as it is unclear whether the presently defined R-state of ATCase would correspond to R or to S in this model. This is because most functional studies of ATCase have been conducted at substrate concentrations lower than those at which substrate inhibition becomes apparent (i.e. the B panels of Figs. 1–4), while most structural studies have been carried out at much higher substrate or ligand concentrations.

Since the simple allosteric models examined can account for the kinetic data alone, other experimental approaches are needed to determine which, if any, simple or more complex [42–44] models are applicable. Simple tests and existing data discriminated against the nested model of Eq. (2), but evaluating other models will require further examination of the substrate-inhibited region of ATCase's activity spectrum. Direct binding studies designed specifically to address the mechanism of substrate inhibition are needed, as are crystallographic experiments, or closer analysis of existing crystallographic data aimed at identifying inhibitory binding sites or demonstrating that they do not exist. Methods that allow binding

and kinetics to be more carefully studied in solution at high aspartate concentrations in solution are also needed. Ultimately, parallel kinetic, binding, and conformational studies will be required to discriminate among possible models.

Acknowledgements

We thank Dr Evan Kantrowitz (Boston College) for supplying plasmids for ATCase production, and we thank H. Morizono, C. Listrom, B. Rajagopal, M. McCann, and M. Tuchman at the University of Minnesota for the data in Fig. 4. This work was supported by NIH Grant DK-17335 (to N.M.A.).

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