15. Kinetic Approaches to the Study of Enzyme Regulation

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The survival of an organism depends on the delicate integration of the activities of myriad biochemical pathways. The maintenance of homeostasis in the face of diverse external and internal stimuli enables the organism to achieve economy in the utilisation of energy and materials by modulation of strategically placed enzymes. Our laboratory has been concerned over the past few years in understanding the mechanism of regulation of aspartate transcarbamylase (ATCase) and glutamine synthetase (GS) two important regulatory enzymes. This review highlights the scope of kinetic methods in the study of the regulation of: (a) plant aspartate transcarbamylase by allosteric interaction and hysteresis; and (b) plant and fungal glutamine synthetase by multiple interactions with metabolites and metal ions.

Regulation of Aspartate Transcarbamylase

1) Properties of Escherichia coli ATCase

Aspartate transcarbamylase (EC 2.1.3.2) catalyses the first committed step in the pathway for the biosynthesis of pyrimidine nucleotides, namely the carbamylation of aspartate by carbamyl phosphate to yield carbamyl aspartate. This enzyme from Escherichia coli has served as a model allosteric protein. The enzyme shows positive homotropic interactions with the substrates, negative heterotropic interactions with CTP, an end product of the pyrimidine biosynthetic pathway and positive heterotropic interaction with ATP, a product of the parallel purine biosynthetic pathway [1, 2]. The native enzyme is an oligomeric protein composed of two catalytic subunits of molecular weight (Mr) 100,000 and three regulatory subunits of Mr 34,000. Each catalytic subunit is a trimer consisting of three polypeptide chains each of Mr 34,000 and the regulatory subunit is a dimer of two poly-
peptide chains of Mr 17,000 [3, 4]. The hexameric nature of the enzyme was confirmed by several lines of indirect evidence [4–8] and established unequivocally by X-ray diffraction and electron microscopy [9–11].

Several models have been proposed to explain the kinetic and physicochemical properties of the enzyme in terms of its quaternary structure and subunit interactions. These include: (a) rotational model [12], (b) functional model [13], (c) model of Chan [14], etc. However, recent results of Schachman and his co-workers and of Chan and his co-workers [15–24] are in accordance with the concerted transition model of Monod et al. [25]. In spite of this overwhelming evidence, there is still some doubt about the exact mechanism of regulation [26]. However, much of the data can be approximated to the model of Monod et al. [25].

2) Regulation of Mung Bean ATCase by Allosteric Interactions and Hysteresis

Our laboratory has been studying ATCase from mung bean seedlings [27–35]. The mung bean ATCase, unlike the E. coli enzyme, is an oligomeric protein of Mr 130,000 exhibiting homotropic cooperative interactions with carbamyl phosphate and heterotropic effects with UMP. A distinctive property of the plant enzyme is the presence of hysteresis. Since this is a comparatively rare phenomenon in enzymes a brief description is presented below.

a) Hysteretic regulation of enzymes: Hysteresis can be defined as the ‘lag’ exhibited by a body in responding to an outside force. Hysteresis in enzymology may be defined as the slow response of an enzyme to a rapid change in the concentration of ligands, such as substrates, allosteric effectors etc. The slow response of a hysteretic enzyme manifests itself as either a ‘lag’ or a ‘burst’ in some kinetic property such as the time course of the reaction catalysed by the enzyme. Figure 1 (inset) depicts the theoretical progress curves representing ‘lag’ and ‘burst’ observed in hysteretic enzymes. ‘Lag’ is generally observed when the reaction catalysed by a hysteretic enzyme is initiated by the addition of the enzyme to a mixture of substrate and an activator. A burst is observed when the reaction is initiated by the addition of the enzyme to a mixture of substrate and an allosteric inhibitor. Both lags and bursts observed in progress curves of reactions catalysed by hysteretic enzymes are due to slow rates of structural alterations caused by the binding of the ligand compared to catalytic rates in the enzyme reaction.

Three types of molecular mechanisms have been propounded to explain the phenomenon of hysteresis in enzymes [36, 37]: (i) slow displacement of one allosteric effector by another either directly or through a conformational change, as in, AMP deaminase (E.C.3.5.4.6) [38]; (ii) ligand induced slow association-dissociation of an enzyme between two forms with differential catalytic activity, could also produce the hysteretic response in an enzyme [39]; (iii) ligand induced slow isomerisation reaction of an inactive or partially
active enzyme species into an active conformation [36, 40, 41]. The theoretical aspects of these three types of mechanisms including diagnostic criteria to distinguish among the three possibilities are discussed by Frieden [37] and Kurganov et al. [41].

Although the physiological significance of hysteresis has not been clearly established, there is speculation that hysteresis is a fine mode of control [36, 40] and could benefit the cell in the following ways: (i) by controlling a sudden flux of a metabolite through a metabolic pathway [42], (ii) by modulating the amplitude of inherent oscillations of a pathway [43–45], (iii) by conferring kinetic cooperativity to a monomeric protein in which site-site interactions are absent [46, 47], and (iv) by altering the kinetic cooperativity in oligomeric enzymes [48]. This has profound physiological significance, for it enables a 'fine control' of inhibition or activation [49]. A list of enzymes
which show both cooperativity and hysteresis is presented in Table 1. Two groups of workers [36, 46] have proposed mechanistic models that link cooperativity and hysteresis.

Table 1. List of enzymes which show both cooperativity and hysteretic response

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Type of cooperativity</th>
<th>Type of transient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threonine deaminase (B. subtilis)</td>
<td>Phosphoenol pyruvate</td>
<td>+ve</td>
<td>lag</td>
<td>50</td>
</tr>
<tr>
<td>D-lactate dehydrogenase</td>
<td>Pyruvate</td>
<td>+ve</td>
<td>lag</td>
<td>51</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>NAD⁺</td>
<td>+ve</td>
<td>lag</td>
<td>52</td>
</tr>
<tr>
<td>Pyruvate kinase (Human erythrocyte)</td>
<td>Phosphoenol pyruvate</td>
<td>+ve</td>
<td>lag</td>
<td>53</td>
</tr>
<tr>
<td>Hexokinase (Yeast)</td>
<td>ATP</td>
<td>-ve</td>
<td>burst</td>
<td>54</td>
</tr>
<tr>
<td>Adeny late deaminase</td>
<td>AMP</td>
<td>+ve</td>
<td>lag</td>
<td>55</td>
</tr>
<tr>
<td>β-Galactosaminidase</td>
<td>Acetyl phosphate</td>
<td>+ve</td>
<td>lag</td>
<td>56</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxy kinase</td>
<td>GTP, oxalo-acetate</td>
<td>-ve</td>
<td>lag</td>
<td>57</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>NADH</td>
<td>+ve</td>
<td>lag</td>
<td>58</td>
</tr>
<tr>
<td>Acetyl coenzyme carboxylase (rat liver)</td>
<td>Mg²⁺, citrate</td>
<td>+ve</td>
<td>lag</td>
<td>59</td>
</tr>
<tr>
<td>Adenylate cyclase (rat liver)</td>
<td>ITP, IDP</td>
<td>+ve</td>
<td>lag</td>
<td>60</td>
</tr>
<tr>
<td>Phosphofructokinase (bovine liver)</td>
<td>Fructose-6-phosphate</td>
<td>+ve</td>
<td>lag</td>
<td>61</td>
</tr>
<tr>
<td>Pyruvate carboxylase (sleep kidney)</td>
<td>Mg-ATP³⁻</td>
<td>+ve</td>
<td>lag</td>
<td>62</td>
</tr>
</tbody>
</table>

b) Hysteresis and cooperativity in mung bean ATCase: Purified mung bean ATCase gave a sigmoid saturation curve with carbamyl phosphate [32]. Using these data, the values of the Hill coefficient (nH) which indicates the strength of interaction between the subunits, L the allosteric constant which is the equilibrium constant between the R and T states of the protein, and c the ratio of the dissociation constants for binding of carbamyl phosphate to R and T states were calculated to be 2.0, 33, 0.008 respectively at pH 6.5 [32]. These values were calculated using an interactive procedure described by Weiker et al. [63] and by using a computer programme written by us which is available on request.
A unique feature of this enzyme was the appearance of a lag phase in the time course of the reaction which increased upon preincubation with UMP and was abolished upon preincubation with carbamyl phosphate (Fig. 1). Preincubation of the enzyme with carbamyl phosphate and starting the reaction with aspartate and UMP resulted in the appearance of a burst phase in the time course (Fig. 1).

It is essential that artifacts of assay etc. are ruled out before embarking on an examination of the mechanism of hysteresis [32, 36, 41]. (i) To ensure that the observed effects were not due to substrate depletion, carbamyl phosphate and aspartate were used at concentrations more than five times the K_m and the product formed in no case was more than 3 to 5 per cent of the substrate(s) added. (ii) There was no inhibition by excess substrate. (iii) The lags could result from artifacts such as product activation and the influence of buffer components of the assay mixture. The products of the reaction, namely carbamyl aspartate and P_i, were shown to be linear, non-competitive and competitive inhibitors of the enzyme when carbamyl phosphate was the varied substrate and had no effect on the time course [28, 29]. Thus, product activation was ruled out as an explanation for the lag. The lag observed upon initiation of the reaction with carbamyl phosphate (Fig. 1) and the burst observed when the enzyme was preincubated with carbamyl phosphate and the reaction started by the addition aspartate and UMP (Fig. 1) are reminiscent of the hysteresic response of allosteric enzymes to activators and inhibitors [37]. Such a behaviour was reported in the case of rat liver phosphofructokinase (E.C.2.7.1.11) [64] and beef liver pyruvate carboxylase (E.C.6.4.1.1) [65]. These considerations along with the observed dependence of the lag phase on the conditions of preincubation clearly suggested for the first time that hysteresis was an inherent property of mung bean AATase and not an artifact of the assay.

Though the lag phase observed in the progress curve (Fig. 1) was obvious, it was further substantiated by the positive intercepts on the time axis of the linear fast phase of the progress curves. The ratio, s/f, of the slope of the initial slow phase of catalysis (s) to that of the final fast phase, (f), was used to quantitate the lag phase. The use of this criterion, however, is subject to a few limitations due to the need for an accurate estimate of the small amount of product formed at the earlier periods of incubation and also due to the assumption that the initial slow phase of catalysis is linear, which is not strictly correct. In view of such limitations, this diagnostic tool was used in this study only to represent the variations of the lag phase with preincubation conditions and alterations due to changes in pH and effector concentrations. This ratio (s/f) is less than 1 for a progress curve with a lag phase, greater than 1 for a curve with a burst phase and equal to 1 for a single linear time course. The s/f values increased from 0.22 at pH 7.5 to 0.95 at pH 9 suggesting that hysteresis was an inherent property of the enzyme and was pH dependent.
The influence of carbamyl phosphate in abolishing and UMP in enhancing the lag phase [32] suggested the slow conversion of the enzyme from inactive or partially active form to an active form. If this hypothesis was valid it could be predicted that the preincubation of the enzyme with higher concentrations of UMP should enhance the lag phase further. This was found to be the case as reflected by the increased lag phase observed in the progress curves when the enzyme was preincubated with increasing concentration of UMP [32]. This was also confirmed by the decreasing values of s/f with increasing concentration of UMP (Table 2). The results discussed above suggested that mung bean ATCase could be a hysteric enzyme.

<table>
<thead>
<tr>
<th>UMP (μM)</th>
<th>V\text{max}\textsuperscript{a}</th>
<th>n\textsubscript{H}</th>
<th>K\text{e+}\textsuperscript{b}</th>
<th>L</th>
<th>s/f</th>
<th>K\text{app}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td>80.0</td>
<td>1.0</td>
<td>0.36</td>
<td>—</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>0</td>
<td>80.0</td>
<td>2.0</td>
<td>0.56</td>
<td>33</td>
<td>0.56</td>
<td>0.96</td>
</tr>
<tr>
<td>125</td>
<td>80.0</td>
<td>2.8</td>
<td>3.98</td>
<td>625</td>
<td>0.212</td>
<td>0.58</td>
</tr>
<tr>
<td>375</td>
<td>81.7</td>
<td>3.2</td>
<td>6.56</td>
<td>6560</td>
<td>0.13</td>
<td>0.38</td>
</tr>
<tr>
<td>500</td>
<td>81.8</td>
<td>3.6</td>
<td>7.50</td>
<td>20730</td>
<td>0.06</td>
<td>0.33</td>
</tr>
</tbody>
</table>

\textsuperscript{a}nmol/min/mg protein; \textsuperscript{b}mM.
\*Preincubated with carbamyl phosphate.

c) \textit{Mechanism of hysteresis in mung bean ATCase}: Slow displacement as mechanism for hysteresis appeared improbable in the case of mung ATCase for the following reasons: (i) several attempts at locating a bound effector were unsuccessful; (ii) the purification of the enzyme involved ammonium sulphate fractionation steps as well as gel filtration on Sephadex G-25 which probably could have removed the bound inhibitor, and (iii) the native enzyme showed a lag in the absence of preincubation with ligands. If one assumes that a bound activator is present and is slowly displaced by the substrate, one would expect a 'burst' and not a lag (Fig. 1).

Another mechanism by which hysteresis is manifested is the association-dissociation of the protein in the presence of a ligand. There is some evidence to suggest that wheat germ ATCase aggregates to higher molecular weight forms in the presence of UMP [66]. It was shown earlier that p-hydroxy-mercuribenzoate (pHMB) dissociated mung bean ATCase and the dissociated form was catalytically active [29]. It was therefore necessary to carefully consider the possibility of association-dissociation as a probable mechanism of hysteresis in this enzyme. A simple diagnostic test for the occurrence of association-dissociation was the dependence of the lag phase on enzyme concentration. If the hypothesis was made that the associated form of the enzyme was more active than the dissociated form, one would expect a decrease in the lag phase with increasing concentrations of the enzyme, whereas an increase in the lag phase would be observed if the
converse of $\omega$'s argument was true. If association-dissociation was not responsible for hysteresis, the lag would be independent of enzyme concentration. The results in Fig. 2 show that the progress curves at different concentrations of the enzyme are superimposable. The identical lag phases suggested that association-dissociation of the enzyme may not be the probable mechanism for hysteresis in mung bean ATCase.

![Graph showing a linear relationship between $P/P_{\text{max}}$ and time (min).](image)

Fig. 2. Time course of the reaction using different amounts of the enzyme. The reaction mixture (12 ml) contained 720 mg (●), or 1.08 mg (△) or 1.44 mg (○) of the enzyme. Carbamyl phosphate (5 mM) and aspartate (10 mM) aliquots (0.8 ml) were withdrawn at different time intervals and assayed as described [32]. The activity at 16 minutes was assumed to be $P_{\text{max}}$ and the product formed (P) at each time interval was expressed as a fraction of this $P_{\text{max}}$.

Although these experiments are sufficiently discriminatory, a finer quantitative tool to rule out association-dissociation is the evaluation of $k_{\text{app}}$ for the slow transition of the enzyme, which would be independent of enzyme concentration in the case of the slow isomerisation mechanism and varies as a function of enzyme concentration in the case of association-dissociation mechanism. The values calculated [45] for progress curves at different enzyme concentrations were 1.0, 0.96, 0.96 at 60, 90 and 120 μg protein respectively indicating that the $k_{\text{app}}$ is independent of enzyme concentration thus providing evidence for the fact that association-dissociation may not be responsible for hysteresis. The evidence discussed above indicates that slow displacement and association-dissociation mechanisms may not be operative in the case of mung bean ATCase, leaving behind only isomerisation as a possible mechanism to explain these results.

The additional evidence for this mechanism was derived from the study of the effects of carbamyl phosphate and UMP on $k_{\text{app}}$. For an isomerisation mechanism, the transition of the enzyme from the initial slow phase of catalysis to the final fast phase is a first order process [50]. A linear first
order plot was obtained in this study supporting the isomerisation hypothesis. The $k_{\text{app}}$ values calculated from these plots for enzyme preincubated with varying concentrations of UMP (Table 2), reaction initiated by different amounts of carbamyl phosphate (Fig. 3) and progress curves of the reaction at various pH [32] were good fits of a straight line, thus providing evidence in support of isomerisation hypothesis. In addition, the identical kinetic constants obtained for aspartate saturation of the enzyme when it was preincubated with carbamyl phosphate alone or when it was preincubated with UMP followed by carbamyl phosphate suggested that carbamyl phosphate was bringing about reversible conformational changes in the enzyme [32].

![Graph showing first order plots for the calculation of $k_{\text{app}}$.](image)

**Fig. 3.** First order plots for the calculation of $k_{\text{app}}$. The enzyme (1.5 mg) was preincubated with 250 $\mu$M UMP for 10 minutes at 26°C. The preincubated enzyme was added to two separate reaction mixtures 12 ml containing either 5 mM (●) or 10 mM (○) carbamyl phosphate. The reaction was initiated by the addition of aspartate. From the time course of the reaction, the first order plot was constructed for the slow transition of the enzyme at the two concentrations of carbamyl phosphate.

Thus, our studies [31, 32] clearly indicate that mung bean ATCase exhibits kinetic hysteresis by a slow isomerisation between two states of the enzyme, R and T, as in the Monod model [25]. The parallel changes in the $n_H$, $s/I$, and $k_{\text{app}}$ values with increasing concentrations of UMP (Table 2), changes in the $n_H$, $s/I$ and $L_0$ values with pH [32] strongly implicate that hysteresis and kinetic cooperativity are probably linked. The implications of such a correlation are that the two phenomena might occur by similar molecular mechanisms probably involving the same binding determinants [40]. This study establishes for the first time a link between kinetic cooperativity and hysteresis in an oligomeric enzyme.
Regulation of Glutamine Synthetase from Mung Bean and *A. niger*

Ammonia, glutamate and glutamine are the key metabolites of nitrogen metabolism in all organisms. Consequently, glutamine synthetase (EC 6.3.1.2) which catalyses the formation of glutamine from glutamate, ammonia and ATP (eq. 1) occupies a central role in the regulation of flow of these metabolites

\[
\text{GS} \quad \text{Glutamate} + \text{ATP} + \text{NH}_4^+ \rightarrow \text{Glutamine} + \text{ADP} + \text{P}_i
\]

(eq. 1)

Mg²⁺

Extensive studies have demonstrated that *E. coli* glutamine synthetase (GS) is under the stringent control by a number of regulatory mechanisms [67–69]. GS is believed to play a key role in the assimilation, storage and translocation of ammonia in higher plants. In leguminous plants that are well-known for their nitrogen fixing capacity, glutamine is an early product and an important metabolite transported from the nodules to other parts of the plant. A study of the regulatory properties of this enzyme isolated from plant sources is of special interest in view of a large number of nitrogenous end products of the secondary metabolism in them. In this context, our laboratory has been interested in the regulation of GS from mung bean (*P. aureus*) seedlings and from *A. niger*. This review highlights some of our work on the regulation of this enzyme. The enzyme from the germinating seedlings of mung bean was purified and characterised [70–72]. The γ-glutamyl transferase reaction catalysed by this enzyme proceeds by a ping pong mechanism [70].

1) *Regulation of Mung Bean GS by Multiple Inhibition*

Regulatory enzymes which catalyse a common step for the biosynthesis of a variety of end products have often evolved mechanisms by which they are only partially inhibited even at high concentrations of a single end product. GS is a common enzyme for the biosynthesis of several amino acids like alanine, glycine, histidine, nucleotides, amino sugars, etc. We have used simple kinetic approaches to elucidate the mechanism of regulation of GS by these metabolites [73, 74].

Alanine is a partial noncompetitive inhibitor while histidine is a partial competitive and glycine is a partial mixed type inhibitor of the biosynthetic activity of GS while glutamate is the varied substrate (Fig. 4). Partial inhibitions are characterised by: (i) a hyperbolic plot, reaching a limiting finite value of velocity when the initial velocity is plotted against the inhibitor concentration at a fixed level of the substrate—in the case of complete inhibition, the velocity decreases to zero; (ii) the replots of the slope and intercept values obtained from the Lineweaver-Burk for the substrate in the presence of different fixed concentrations of a partial inhibitor is hyperbolic, while for a complete inhibitor such plots are either linear or parabolic; and (iii) the intercept on the ordinate of a double reciprocal plot of fractional in-
Fig. 4. Kinetics of inhibition of mung bean GS by amino acids (a) Mixed type inhibition by glycine. The reaction mixture contained imidazole hydrochloride buffer (80 mM), pH 7.2, MgCl₂ (10 mM), 2-mercaptoethanol (10 mM), ATP (10 mM), NH₄OH (10 mM) and the enzyme which had been preincubated for five minutes at 37°C with the concentrations of glycine shown in the figure. Glutamate concentration was varied between 1-50 mM and assayed for product, γ-glutamyl hydroxamate formed as described [72]. Double reciprocal plots: glycine 0 mM (●) 30 mM (○) and 50 mM (▲).

(b) Noncompetitive inhibition by alanine—the enzyme was assayed as described above, with glutamate concentration varied in the range 0.5 to 50 mM at alanine 0 mM (●) 30 mM (○), 50 mM (▲).

(c) Competitive inhibition by histidine. The enzyme was assayed as described above except that glutamate concentration was varied in the range 1-50 mM at histidine 0 mM (●), 150 mM (○) and 175 mM (▲).
hibition against the inhibitor concentration (that is, $1/i$ vs $1/(1)$ where $i = V_0 - V_i/V_0$, $V_i$ is the the velocity of the reaction at a fixed substrate concentration in the presence of the inhibitor and $V_0$ is the velocity of the reaction at the same substrate concentration in the absence of the inhibitor) is always greater than unity in the case of a partial inhibitor—all other types of inhibition give a value of 1 [75].

The partial inhibition can be fitted into the models of Stadtman et al. [76] and Segel [75]. In the model of Stadtman et al. [76], the enzyme possesses two distinct and mutually exclusive inhibitor sites. When the inhibitor binds to one of the sites (the 'inhibitory' site), it induces a conformational change in the enzyme that either prevents the substrate from binding to the catalytic site or prevents the catalytic activity even though the substrate is bound to the enzyme. The binding of the inhibitor to the other inhibitor site (the 'noninhibitory site') is also prevented. When the inhibitor binds to the noninhibitory site, binding of the inhibitor to the inhibitory site is prevented but there is no effect on the catalytic activity of the enzyme. Although our data [73] on partial inhibition of GS can be adequately explained by the above model, alternate explanations cannot be ruled out.

In the model of Segel [75] it is assumed that the inhibitors bind to a single specific site to yield enzyme-inhibitor and enzyme-substrate-inhibitor complexes. The enzyme-substrate-inhibitor complex can yield products with equal or less facility than the enzyme-substrate complex. The partial competitive inhibition by histidine, noncompetitive inhibition by alanine and mixed type inhibition by glycine observed with the mung bean enzyme [73] can be explained using this model.

The observations that several compounds, structurally unrelated to substrates or products, inhibit the activity of a regulatory enzyme suggest that multiple sites for the binding of these effectors exist on the enzyme. Multiple inhibition analysis is an important kinetic tool which provides information on: (i) mosaic of sites for an inhibitor, (ii) a single site capable of binding several inhibitors, and (iii) multiple sites each specific for an inhibitor. This kinetic approach helps in establishing the mutual exclusivity or nonexclusivity of the inhibitor sites. Two ligands are said to be mutually exclusive when the binding of one inhibitor precludes the binding of the other. Nonexclusivity of the inhibitor binding sites arises when the inhibitors are independent in their action. One of the diagnostic tests for establishing the mutual exclusivity or nonexclusivity is provided by the Dixon plot of $1/v$ versus the concentration of one inhibitor at a constant concentration of the substrates and at different fixed concentrations of the other inhibitor. Such plots yield parallel lines in the case of mutually exclusive inhibitors, while for non-exclusive inhibitors, intersecting pattern of lines are obtained.

The data on multiple inhibition of GS by alanine and glycine (Fig. 5) suggest nonexclusivity of their binding sites. The equilibria shown in Fig. 5(a) explain the presence of separate and noninteracting binding sites for
glycine and alanine, and the partial noncompetitive and mixed type inhibition by these inhibitors.

![Graphs showing inhibition of enzyme activity by glycine and alanine](image)

**Fig. 5.** (a) Inhibition of mung bean glutamine synthetase activity by glycine in the presence of alanine. The enzyme assays were carried out as described in the legend for figure 4(a) except that the enzyme was preincubated with alanine 0 mM (●), 30 mM (○) and 50 mM (▲) at varying concentrations of glycine and assayed for activity at saturating concentrations of substrates.

Inset: The reciprocal of the velocity computed assuming equilibria shown in Fig. 5(b) and the constants determined are plotted against the concentration of glycine. The very good correlation between the observed velocity and the computed velocity supports the model given in (b).

(b) The equilibria among enzyme species in the presence of the non-competitive inhibitor alanine (I) and the mixed type inhibitor glycine (X). S represents...
Similar lysis was carried out with other pairs of amino acids and nucleotide inhibitors and the kinetic mechanism of their inhibitors was established [73]. The nonexclusivity of these inhibitors was further confirmed by cumulative inhibition studies.

Cumulative inhibition is observed when the inhibitors are independent in their action. The enzyme activity in the presence of a mixture of inhibitors is equal to the product of the fractional activities with each inhibitor. The cumulative response to pairs of inhibitors is given by eq. 2.

$$T_i = I_i + (100 - I_i) X_i/100$$  \hspace{1cm} \text{(eq. 2)}$$

where $I_i$ represents the percent inhibition with inhibitor $I_i$, and $X_i$, the percent inhibition by $X_i$, and $T_i$, the percent inhibition in the presence of $I$ and $X$.

Cumulative inhibition observed with alanine, glycine and histidine support the contention that these amino acids possess separate and nonexclusive binding sites. This also suggests that the binding of one effector has no effect on the binding of the other. Cumulative inhibition is also observed when the amino acids interacted with AMP and ADP indicating that the binding sites for these nucleotides are different from the amino acid binding sites [73].

The results presented on mung bean GS indicate that adenine nucleotides and amino acids regulate the activity of the enzyme and the enzyme possesses separate sites for the regulators. The existence of multiple sites for interaction of different products and the interplay of the effects of these inhibitors on the activity of GS, enables it to respond uniquely to changes in the concentrations of these metabolites.

2) Regulation of A. niger GS by Metal Ion Activation

In our exploration of other types regulatory mechanisms operating on GS, we examined the enzyme from A. niger and demonstrated that although the activity was not modulated by end products of the pathway, it was affected by interaction at multiple sites with Mn$^{2+}$ and Mg$^{2+}$ [77, 78].

Production of citric acid by fermentation using this fungus continues to be a preferred commercial process. A derangement of carbohydrate metabolism leading to excretion of citric acid into the medium by A. niger is extensively used for the production of this compound by fermentation. The conditions of fermentation, namely low pH, high amounts of carbon source and essentially Mn$^{2+}$ deficient medium [79] resulting in elevated intracellular NH$_4^+$ pools [80], accumulation of L-glutamate, L-glutamine and amino acids derived from it [81], suggested to us that the metabolic interlock between glutamate and $E$ represents the enzyme in the presence of saturating concentrations of ATP and hydroxylamine. $a$ is the factor by which $K_{aE}$ changes when $X$ occupies the enzyme. $K_I$ and $K_S$ are the constants for the dissociation of EI and EX respectively. $a K_s$ is the constant for the dissociation of ESX and ESIX complexes. $\beta_1$, $\beta_2$, and $\beta_3$ are the factors by which the rate of the uninhibited reaction is reduced in the presence of the inhibitors. $K_{I(aET)} = 21$ mM, $K_{S(aET)} = 22$ mM, $a = 3.0$, $\beta_1 = 0.53$, $\beta_2 = 0.137$, $\beta_3 = 1.0$. 

carbon and nitrogen metabolism may have been deranged at the GS step.

The biosynthetic reaction of *A. niger* GS required either Mg²⁺ or Mn²⁺ as metal ion activator and ATP as the nucleotide substrate (eq. 1). Detailed and quite complicated procedures have been evolved to carry out kinetic studies on metal-activated enzymes. Qualitative criteria for distinguishing between different effects of the activator metal ion and the nucleotide substrates have been formulated by Mildvan [82], and more recently by Morrison [83]. A general rapid equilibrium model for a unireactant enzyme that combines with nucleotide substrate, metal ion and metal-nucleotide complex (true substrate of the enzyme) was presented by London and Steck [84]. This kinetic model can be adapted with suitable restrictions and modifications [85] to describe a more complex system like glutamine synthetase.

Possible interaction of metal ions and the nucleotide with each other and also with the enzyme were examined by studying the saturation pattern of metal ions (Mg²⁺ or Mn²⁺) by measuring the initial velocity at different, fixed concentrations of ATP, or by varying the concentration of ATP at different fixed concentrations of the metal ion (either Mg²⁺ or Mn²⁺). These saturation curves are identified by the varied ligand, that is, when ATP is varied at different fixed concentrations of Mg²⁺ the saturation pattern is known as “ATP-profiles” and “Mg²⁺-profiles” describe the saturation pattern when Mg²⁺ was varied at different fixed concentrations of ATP. Similarly “Mn²⁺-profiles” are defined. Two graphical methods have been described [84] to evaluate various kinetic parameters of a system where Me-ATP complex, free Me (Mg²⁺ or Mn²⁺) and free ATP combine with the enzyme. Both the methods make use of the “approximation of Me. ATP complex”, using $K_0$

the ligand equilibrium ATP+Me=Me. ATP.

*Kinetics of Mg²⁺ activation*: Mg²⁺ and ATP profiles for *A. niger* GS are presented in Fig. 6. All the Mg²⁺-profiles were sigmoid and their sigmoidicity increased with increasing concentrations of ATP. Also, there was no inhibition at higher concentrations of Mg²⁺. Maximal activity was obtained when Mg²⁺ and ATP ratio was about 2 : 1. There was no apparent sigmoidicity in the ascending limbs of ATP-profiles but after the peak of activity was reached, increasing concentrations of ATP caused progressively larger inhibition of enzyme activity. The maximal velocity in each case was different and was dependent on the fixed concentration of Mg²⁺. The peak positions were at Mg²⁺: ATP ratio of approx. 2 : 1. When Mg²⁺ was present in excess (40 mM) over ATP (10 mM), the ATP-profile was hyperbolic. A $K_m$ value of 1.5 mM was obtained for Mg²⁺-ATP complex from a double-reciprocal plot [78].

As Dixon plots for the inhibition of ATP were non-linear, the $K_i$ values for ATP were obtained by the isovelocicity method. It is evident from Fig. 6b that the same velocity (isovelocity) is obtained at two different concentrations of ATP for each Mg²⁺ concentration. These two sets of extrapolated ATP
Fig. 6. Saturation of A. niger Mg\(^{2+}\) dependent glutamine synthetase activity by Mg\(^{2+}\) and ATP.
(a) Mg\(^{2+}\) profiles: The reaction mixture (0.5 ml) contained imidazole hydrochloride (100 mM), pH 7.8, NH\(_4\)OH (50 mM) and L-glutamate (100 mM). Mg\(^{2+}\) concentration was varied in the range 0-100 mM at 5, 10, 15 and 20 mM ATP. The reaction was started by the addition of enzyme (9.6 µg).
(b) ATP profiles: The enzyme assays were carried out in the manner described above with ATP concentrations varied in the range 0-30 mM at 5, 8, 12, 20 and 40 mM MgCl\(_2\). Broken horizontal lines indicate the isovelocity points. Arrows indicate change in sigmoidicity with increasing fixed concentrations of the substrate.

concentrations were plotted against the corresponding concentration of Mg\(^{2+}\) according to London and Steck [84]. At low ATP concentrations (Mg\(^{2+}\) excess region), the isovelocity replots were linear and parallel to Mg\(^{2+}\)-axis. On the other hand, at higher ATP concentrations, a set of intersecting lines was obtained and the point of intersection on the ATP axis gave a K\(_{ATP}\) value of 2.4 mM.

Kinetics of Mn\(^{2+}\) activation: The Mn\(^{2+}\)-profiles were bell shaped with sigmoid ascending lines (Fig. 7a). The position of the peak velocity was dependent upon the fixed concentration of ATP and maxima were reached at Mn\(^{2+}\) : ATP ratio of about 1 : 2. The sigmoidicity of the ascending limbs increased with increasing fixed concentration of ATP. Unlike Mg\(^{2+}\)-profiles (Fig. 6), the Mn\(^{2+}\)-profiles showed a distinct inhibitory phase.

The ATP-profiles for the Mn\(^{2+}\)-supported synthetase assay were once again bell shaped with sigmoid ascending limbs (Fig. 7b). The sigmoidicity was more apparent at higher fixed concentrations of Mn\(^{2+}\) and the peak velocity occurred when Mn\(^{2+}\) : ATP ratio was about 1 : 2. The constants K\(_{ATP}\) (1.2 mM), K\(_{Mn}\) (0.7 mM) and K\(_{Mn,ATP}\) (0.9 mM) were obtained by isovelocity analysis of the data. The concentrations of Mn\(^{2+}\) and ATP at the peak
Fig. 7. (a) Mn²⁺ and ATP saturation of A. niger Mn²⁺ dependent glutamine synthetase activity.

Mn²⁺ profiles: The reaction mixture was similar to that described in the legend to Fig. 6 except Mn⁶⁺ was replaced by MnSO₄ in the concentration range 0-20 mM at 4, 7, 10 and 13 mM ATP. The reaction was started by the addition of enzyme (16 μg).

(b) ATP profiles: The reaction mixtures were the same as described above except that ATP concentrations were varied in the range 0-25 mM at 1, 2, 3 and 6 mM MnSO₄. Broken horizontal lines represent the isovelocity points and the arrows indicate shift of curves with increasing fixed concentration of the ligand.

For each of the ATP-profiles and Mn²⁺ profiles were plotted as coordinate axes (called 'locus of the peak velocities'). Such an analysis of ATP-profiles and Mn²⁺-profiles gave Kₒ value of 74 μM.

Independent evidence for the interaction of Mn²⁺ with the enzyme was obtained by monitoring the protection afforded by low concentrations of Mn²⁺ against inactivation of the enzyme by N-ethylmaleimide and phenyl glyoxal. Kₘ₅ₓ₂⁺ values of 57 μM and 14 μM were calculated from these protection experiments indicating that there are probably more than one class of Mn²⁺ binding sites as the Kₘ₅ₓ⁺ (0.6 mM) calculated by kinetic methods was at least an order of magnitude higher. Based on the kinetic
constants defined (Table 3) as described above as well as our earlier observations [77, 82], a model to explain the interaction of Mn$^{2+}$, Mg$^{2+}$ and ATP with A. niger GS is presented in Fig. 8. The forward triangle of the

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kinetic constant* (mM)</th>
<th>Dissociation constant$^b$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$-ATP</td>
<td>1.5</td>
<td>0.9</td>
</tr>
<tr>
<td>ATP</td>
<td>2.4</td>
<td>0.052</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>x</td>
<td>0.014</td>
</tr>
<tr>
<td>Mn$^{2+}$-ATP</td>
<td>0.9</td>
<td>0.074$^d$</td>
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<tr>
<td>ATP</td>
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<td>0.021</td>
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<tr>
<td>Mn$^{2+}$</td>
<td>0.6</td>
<td>0.014$^c$</td>
</tr>
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</table>

$^a$Kinetic constants were obtained from saturation plots.

$^b$Dissociation constants were obtained by measuring the protection afforded by the ligands against inactivation of the enzyme by phenylglyoxal.

$^c$From protection experiments using NEM as the inactivating agent.

$^d$Dissociation constant for the dissociation of Mn$^{2+}$-ATP complex into Mn$^{2+}$ and ATP was calculated from replots of locus of peak velocities.

\[
\text{Me} + \text{ATP} \xrightarrow{K_0} \text{Me} \cdot \text{ATP}
\]

Fig. 8. Equilibria for the interaction of the metal ion Me (Mg$^{2+}$ or Mn$^{2+}$) and ATP with A. niger glutamine synthetase.
bottom face of the cube represents the equilibria for Mg$^{2+}$-supported activity and the entire bottom face represents the equilibria for the Mn$^{2+}$-dependent activity of A. niger GS. It should be emphasized that this mechanism does not make any assumptions regarding the order of binding of other substrates (NH$_4^+$ and glutamate) or release of products. The n$_1$ sites represent the high affinity binding of free metal ion (Mn$^{2+}$) and n$_2$, the low affinity site, where Mn-ATP complex binds as one of the substrates. This mechanism is represented by the top face of the equilibrium cube. Tight and weak binding sites for metal ions have also been observed in the case of E. coli GS.

The K$_{m}$ values for Mn$^{2+}$-ATP and Mg$^{2+}$-ATP agree well with a recent estimate [86] of the intracellular concentration of ATP (1 mM) in A. niger. In view of the high affinity site for Mn$^{2+}$ (n$_1$ site) and the availability of sufficient amounts of Mn$^{2+}$ within the A. niger cells[87], it could be postulated that A. niger enzyme occurs naturally as an Mn$^{2+}$-protein. It is probably for this reason that an Mn$^{2+}$ deficient medium is used for citric acid fermentation. Further under the conditions of citric fermentations such as low pH, Mn$^{2+}$ deficiency, high concentration of carbon source, GS activity is almost completely inhibited. This block leads to decreased biosynthetic potential and backup of the tricarboxylic acid cycle intermediates resulting in citric acid excretion by the fungus[88].

It is evident from this review that the same enzyme is regulated by widely different mechanisms in different organisms highlighting the concept that the regulatory function is more constant than the regulatory mechanism.

Summary

A variety of mechanisms operate to regulate the activity of key enzymes in metabolic pathways. This is achieved by the interaction of effector molecules which cause changes in equilibrium between states of the protein, association-dissociation, binding at multiple sites and hysteresis. This review highlights the use of kinetic approaches in study of enzyme regulation. Mung bean aspartate transcarbamylase is regulated by UMP and carbamyl phosphate binding with differential affinity to the two states of the protein in equilibrium. Another novel feature of the regulation of this enzyme is the slow isomerization of the enzyme upon binding of effectors leading to hysteresis.

Hysteresis in this enzyme is linked to cooperativity as evidenced by the change in the Hill coefficient, k$_{app}$ values etc. On the other hand mung bean glutamine synthetase, although not exhibiting cooperativity in the binding of ligands, is regulated by amino acids and nucleotides which interact in a nonexclusive manner at multiple sites on the enzyme. However, in the case of glutamine synthetase from A. niger, the regulation appears to be related to the concentration of metal ions (Mg$^{2+}$ and Mn$^{2+}$) in the medium. This regulation of glutamine synthetase may have a significant role in the disruption of the metabolic interlock between carbohydrate and nitrogen metabo-
lism leading the backup of tricarboxylic acid cycle intermediates during citric acid fermentation by *A. niger*.

**Abbreviations**

ATCase, aspartate transcarbamylase; GS, glutamine synthetase; Mr, molecular weight; nH, Hill coefficient; L, allosteric constant; R, relaxed state of the protein with greater affinity to the substrate; T, taut state of the protein with greater affinity to the allosteric negative effector; c, ratio of the dissociation constants for effectors to the R and T states; K_m, Michaelis constant; s, slope of the initial slow phase of catalysis; f, slope of the final fast phase; k_{p1}, pseudo first order rate constant; Me, metal ion; K_i, dissociation constant for the enzyme inhibitor complex; K_a, association constant for the formation of the metal ATP complex; V_{max}, velocity at infinite concentration of the substrate; NEM, N-ethyl maleimide.

**REFERENCES**

1979.