moved up and down. The fluctuations suggested by Fig. 5 would require a complex motion of the ocean floor over broad areas. These are more easily explained by assuming that the only vertical motion of points on the ocean floor has been downward, as they moved away from the crest of the Mid-Atlantic Ridge, but that the zone of calcium carbonate compensation has fluctuated through considerable distances in the water, owing to paleogeographic factors (changing interconnections among basins) and paleooceanographic factors (changes in chemical composition, circulation, and biological productivity). During the later Tertiary, this fluctuation would be of the order of 1 kilometer. An extreme fluctuation is postulated for the end of the Cretaceous–early Paleocene, at which time the two compensation levels indicated here are thought to have risen well into the photic zone (*33*).

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Regulation of Enzyme Activity

The activity of enzymes can be controlled by a multiplicity of conformational equilibria.

Gordon G. Hammes and Cheng-Wen Wu

The living cell seldom either synthesizes or degrades more material than is necessary for normal metabolism and growth. In fact all major metabolic pathways contain the capacity for self-regulation. The control of cellular metabolism ultimately involves the regulation of enzyme activity. In broad terms, enzymes can be regulated in two ways: genetic control and direct control of catalysis. Some of the regulatory mechanisms are illustrated in Fig. 1.

In many microorganisms, as well as in a few mammals, the addition of a substrate has been found to induce the synthesis of an enzyme which reacts with this particular substrate; conversely some compounds can cause the repression of enzyme synthesis. Both induction and repression of enzyme synthesis act at the genetic level, and the biochemical and genetic hypotheses involved have been extensively reviewed (*1*).

Direct control of enzyme activity can occur either through the catalytic mechanism itself or through a coupling of the catalytic mechanism with other processes. An example of the former case is simply the Michaelis-Menten kinetics characterizing enzymatic reactions: As the substrate concentration increases, the reaction rate increases until a limiting value is reached; moreover, as the product accumulates, the reaction rate decreases. A more subtle case is where one compound serves as a substrate for more than one enzyme: At low concentrations of substrate, reaction occurs with the enzyme for which it has a higher affinity, and at high concentrations it reacts with the second enzyme, enhancing the dissipation of the substrate. For many enzymes, coenzymes are necessary for catalysis. Since small amounts of coenzymes exist in the cell relative to the number of enzymatic reactions in which they are involved, the concentrations of these coenzymes could have a control function. Normally the binding of substrate to enzyme follows a hyperbolic isotherm, but in enzymes with multiple subunits the binding isotherm may become sigmoidal

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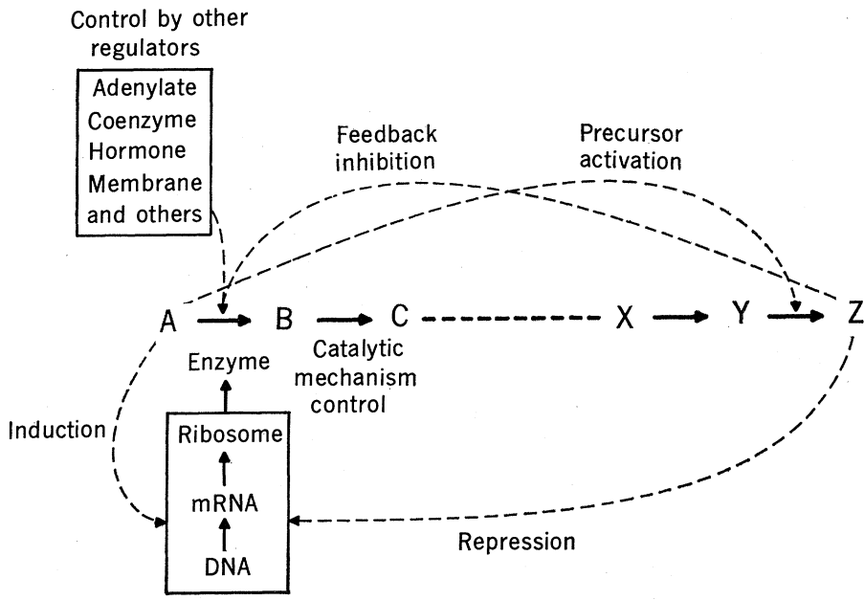


Fig. 1. Schematic representation of mechanisms for the regulation of enzyme activity.

owing to the effect of subunit interactions on substrate binding (Fig. 2). It can be seen that a sigmoidal binding curve has a region where the reaction rate is much more sensitive to the substrate concentration than is the case for a hyperbolic isotherm; therefore the rate of the enzymatic reaction can be closely regulated by the concentration of the substrate.

The control of enzyme activity by coupling with other processes usually implies regulation by ligands which do not participate in the catalytic process and, in fact, are often structurally unrelated to the substrate. The major types of regulatory mechanisms of this category can be classified as follows.

1) Feedback inhibition. In this case the regulatory ligand is the end product of a metabolic pathway which can shut off its own formation by inhibiting the activity of one of the early enzymes on its own synthetic pathway. For example, threonine deaminase, the first enzyme in the biosynthetic pathway for isoleucine, is strongly inhibited by isoleucine even through isoleucine is not a substrate or product of the enzyme (*2*).

2) Precursor activation. The regulatory ligand is the first metabolite of a pathway and activates the last enzyme of the sequence. For example, mammalian glycogen synthetase is activated by glucose 6-phosphate, a precursor of glycogen (*3*).

3) Polymerization-depolymerization. The association-dissociation reactions of a protein (for instance, multisubunit enzymes or multienzyme complexes), as triggered by changes in protein concentration or ligand binding, can alter the enzyme activity. Such reactions may be either rapid or slow and can lead to sigmoidal binding of substrates (*4*).

4) Energy-link control. The regulatory ligands are adenylates or other purine or pyrimidine nucleotides that may serve as indicators of the energy state of the cell. Energy in the cell is generated in the form of adenosine triphosphate (ATP), which is utilized in biosynthetic pathways with the production of adenosine diphosphates (ADP) and monophosphates (AMP). The enzymatic reactions involved in energy generation are activated by ADP or AMP when the energy supply is low, and inhibited by ATP when the energy supply is high (*5*).

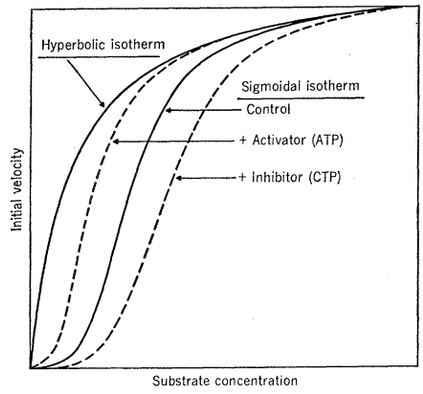


Fig. 2. Hyperbolic and sigmoidal binding isotherms, and the effect of allosteric activators and inhibitors on the sigmoidal isotherm. Adenosine triphosphate (ATP) is an activator and cytidine triphosphate (CTP) an inhibitor for aspartate transcarbamylase from *E. coli* as described in the text.

5) Hormone control. The regulatory ligand is a hormone, which often regulates metabolism through a complex mechanism. For example, it can regulate the activity of adenyl cyclase, and the cyclic AMP produced regulates many metabolic processes (*6*).

Many other control mechanisms are known to exist: For example, modulation of enzyme activity through chemical modification of the enzyme and through binding to membranes and macromolecules. Although all of these mechanisms are of importance, in this article we consider only the molecular basis of sigmoidal binding curves and the control exerted by metabolites which are not themselves substrates or products of the particular enzymatic reaction (allosteric regulation). After we discuss some of the models proposed to account for allosteric control, we present the particular case of the enzyme aspartate transcarbamylase (E.C. 2.1.3.2) in order to illustrate the information concerning the mechanism of action which can be obtained through detailed kinetic studies.

Molecular Mechanisms of Allosteric Control

Allosteric effects (*7*) are defined as indirect interactions between topographically distinct binding sites mediated by the protein molecule through conformational changes. Heterotropic interactions are those which occur between dissimilar ligand molecules and are typified by the effect of activators and inhibitors on enzyme activity. Homotropic interactions are those which occur between identical ligand molecules and may be expressed by sigmoidal binding isotherms of the ligand in question.

Two limiting molecular models have been proposed to account for the allosteric control mechanisms. One is due to Monod, Wyman, and Changeux (MWC model) (*8*); the other is due to Adair, Koshland, Nemethy, and Filmer (AKNF model) (*9*). Both are based on the subunit structure of proteins and alterations in conformation coupled to ligand binding.

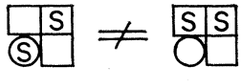
The MWC model is schematically

Fig. 3. Allosteric models of Monod, Wyman, and Changeux (MWC) and of Adair, Koshland, Nemethy, and Filmer (AKNF) for a four-subunit enzyme. The squares and circles are different conformations of the subunits, and S is the substrate.

illustrated for a four subunit enzyme in Fig. 3. This model is based on three assumptions. (i) The enzyme consists of two or more identical subunits, each containing a site for the substrate or modifier; (ii) at least two different conformational states (usually designated as R and T states) are in equilibrium and differ in their affinities for substrate or modifier; and (iii) the conformational changes of all subunits occur in a concerted manner (conservation of structural symmetry). In the absence of ligand, the enzyme exists largely in T states, but substrate binds preferentially to R states, so that the conformational equilibrium is shifted by the binding of the substrate, which can lead to sigmoidal saturation curves. The allosteric inhibitors or activators bind preferentially to the T or R states, respectively, and thus can reduce or enhance the sigmoidicity of the substrate saturation curve, as shown in Fig. 2.

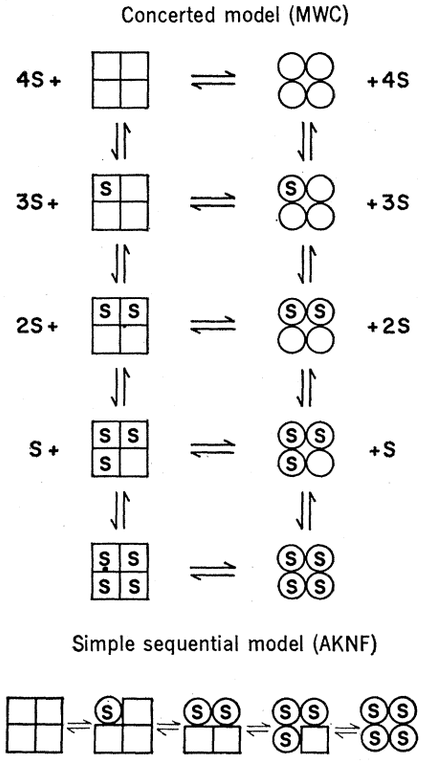
The AKNF model postulates a sequential change of the subunit conformation as each ligand (substrate or modifier) is bound. This is also shown schematically in Fig. 3. In this model a ligand induces the change of conformational state of one subunit, which affects the binding of the next ligand or of a different ligand through the change in the subunit interactions.

Both the AKNF and MWC models are limiting cases of a more general scheme (Fig. 4). Actually the situation is even more complex than that shown in Fig. 4 because states with more than a single ligand bound can take up different geometrical configurations; for example



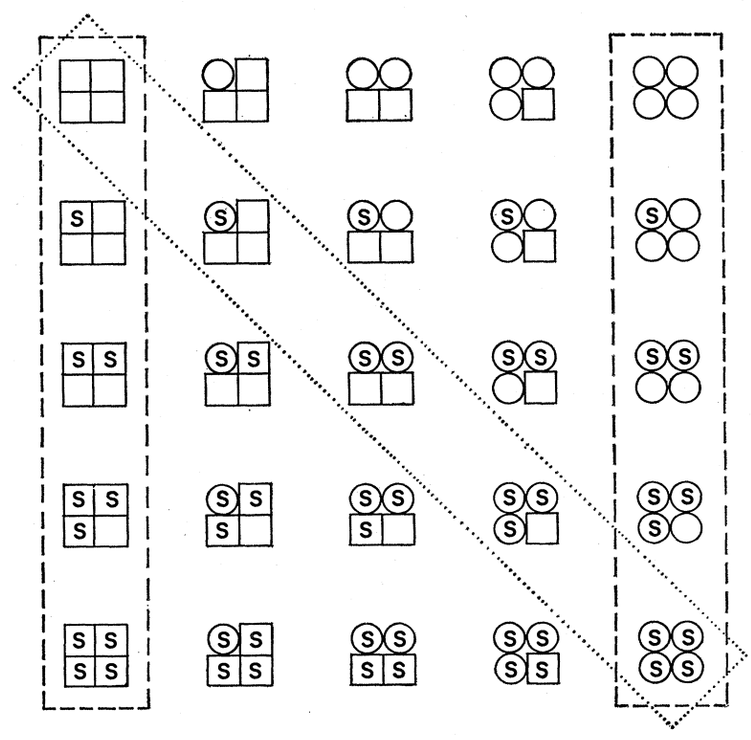
Thus these two limiting models are approximations of a more complex mechanism of subunit interactions.

Fig. 4. A general allosteric model for the binding of substrate, S, to a four-subunit enzyme. The squares and circles are different conformations of the subunits. The MWC model is shown by dashed lines and the AKNF model by dotted lines. The free substrate and arrows between the states are omitted for the sake of clarity.



Attempts to distinguish between the two models have centered on the fact that the MWC model predicts a concerted conformational change, while the AKNF model predicts a sequential change of conformation for each subunit. This means that the extent of binding of ligand and of the change in conformation should show a linear relationship in the latter case but not in the former. Also the homotropic effects are always positive for the MWC model (that is, the binding of the first molecule *enhances* the binding of the second), whereas it may be positive or negative in the AKNF model. Finally, the MWC model assumes that the same conformational state is stabilized by a variety of ligands, whereas the AKNF model predicts that different ligands might induce different conformational changes.

Many different techniques have been used in attempts to elucidate allosteric mechanisms: kinetics, equilibrium dialysis, spectroscopy, ultracentrifugation, osmometry, tryptic digestivity, sulfhydryl reactivity, spin labels, and others. However, most of the results have been quite ambiguous, primarily because only overall changes are observed, which may be related to catalysis, control, or both. Ideally in order to understand the control mechanism, these overall changes should be broken down to their elementary steps. In principle, this is possible by spreading out the processes on the time axis. This involves the measurement, by means of relaxation techniques, of very fast reaction rates. Using this approach, we have studied the regulatory mechanism for aspartate transcarbamylase of *Escherichia coli*.



Aspartate Transcarbamylase of Escherichia coli

Aspartate transcarbamylase is the first enzyme in the pathway of pyrimidine biosynthesis and catalyzes the formation of carbamyl-L-aspartate from L-aspartate and carbamyl phosphate. The enzyme is subject to inhibition by cytidine triphosphate (CTP), the end product of the pyrimidine pathway (*10, 11*). Furthermore, ATP is an activator of the enzyme and an effective antagonist of the inhibitory action of CTP (*11*); this may serve to regulate the relative rates of synthesis of purine and pyrimidine nucleotides, which are required in approximately equal quantities for nucleic acid synthesis.

The kinetic properties of aspartate transcarbamylase are characteristic of a regulatory enzyme. The saturation of the enzyme by L-aspartate at high carbamyl phosphate concentrations (*11*), as well as by carbamyl phosphate at high aspartate concentrations (*12*), is sigmoidal and suggests cooperative binding of these two substrates. The effectors CTP and ATP do not alter the maximum velocity of the enzymatic reaction but shift the position of the substrate saturation curve, which appears more sigmoidal in the presence of CTP and less sigmoidal in the presence of ATP, as is indicated schematically in Fig. 2. Moreover, the effect of CTP is not exerted by direct competition with a substrate for binding at the active site. This is most dramatically shown by the fact that, when the native enzyme is treated with mercurials, two types of subunits are obtained (*13*). The larger subunit (catalytic subunit) is enzymatically active but is insensitive to allosteric effectors, whereas the smaller subunit (regulatory subunit) binds allosteric effectors but is catalytically inert. Recombination of the separated subunits gives almost complete restoration of the original allosteric properties of the enzyme. The native enzyme (molecular weight 310,000) contains six catalytic subunits (molecular weight 33,000) and six regulatory subunits (molecular weight 17,000) (*14*). Although the enzyme has 12 polypeptide chains, for the purpose of the models previously discussed the fundamental "subunit" is generally regarded as containing one regulatory and one catalytic site so that the native enzyme has six such subunits. However, the validity of this assumption remains to be proved.

Because of the availability of large quantities of purified enzyme, numerous studies have been made of the control mechanism of aspartate transcarbamylase. Equilibrium dialysis, sedimentation velocity experiments, and sulfhydryl reactivity (*15*) have been analyzed in terms of a concerted (MWC) model. On the other hand, McClintock and Markus (*16*) have found that the model best describing the tryptic digestivity and sulfhydryl reactivity depends on which ligands occupy the catalytic site. We have attempted to resolve some of the mechanistic ambiguities by carrying out relaxation experiments to elucidate the elementary steps in the catalytic and regulatory mechanisms.

Table 1. Elementary steps detected with relaxation techniques.

Reactant	Mechanism	Effectors*
	Native enzyme	
BrCTP	Bimolecular association-dissociation	
Carbamyl phosphate	Bimolecular association-dissociation	
BrCTP (10 m*M* succinate, 1 m*M* carbamyl phosphate)	Conformational change (concerted or stepwise)	Carbamyl phosphate, succinate
Carbamyl phosphate (10 m*M* succinate)	Conformational change (stepwise)	
Carbamyl phosphate (10 m*M* succinate)	Conformational change (concerted)	BrCTP
Succinate (1 m*M* carbamyl phosphate)	Conformational change (concerted)	BrCTP
L-Malate (1 m*M* carbamyl phosphate)	Conformational change (concerted)	BrCTP
	Regulatory subunit	
BrCTP	Bimolecular association-dissociation	
	Catalytic subunit	
Carbamyl phosphate	Bimolecular association-dissociation	
Succinate (1 m*M* carbamyl phosphate)	Conformational change (stepwise)	
L-Malate (1 m*M* carbamyl phosphate)	Conformational change (stepwise)	

* Substances that influence the rate process in a manner not due to direct competition for the binding site.

Relaxation Spectra of Aspartate Transcarbamylase

A number of discrete steps have been observed to accompany the binding of substrates and effectors to aspartate transcarbamylase and its subunits (*17–20*). The results obtained are summarized in Table 1 and are considered in some detail below.

Equilibrium and kinetic studies of the interaction of the modifier analog 5-bromocytidine triphosphate (BrCTP) with aspartate transcarbamylase and its regulatory subunit have been performed with the use of difference spectroscopy and the temperature-jump method (*17*). The inhibitor BrCTP rather than CTP was used because, when bound to the enzyme or regulatory subunit, it produces a difference spectrum with a maximum at 308 nanometers so that the absorption of the protein does not interfere with the spectrophotometric detection of binding. A single relaxation process in the time range 0.1 to 1 millisecond was observed for the binding of BrCTP to the enzyme and regulatory subunit with the temperature-jump method. In the absence of substrates, the reciprocal relaxation time ($1/\tau$) increases linearly with the sum of the free enzyme (or regulatory subunit) and free modifier concentration. This is characteristic of the simple binding mechanism

$$E + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EM \qquad (1)$$

where E is the enzyme (or its subunit), M is the modifier (or substrate in the case described later), and EM is the enzyme-modifier complex. The reciprocal relaxation time for this mechanism is given by

$$1/\tau = k_1[(\bar{E}) + (\bar{M})] + k_{-1} \qquad (2)$$

where the overbars designate equilibrium concentrations. Thus when $1/\tau$ is plotted against $[(\bar{E}) + (\bar{M})]$, we obtain a straight line for this mechanism. Moreover, the equilibrium dissociation constant determined kinetically (k_{-1}/k_1) was found to be identical with that determined by difference spectroscopy.

In the presence of saturating concentrations of carbamyl phosphate and succinate (a catalytically inactive aspartate analog), the concentration depen-

dence of the reciprocal relaxation time is unchanged for the regulatory subunit, but changes markedly for the native enzyme. It increases with increasing free concentrations of enzyme and effector, but approaches a constant value at high values of $[(\bar{E}) + (\bar{M})]$. Since this effect is observed only with the native enzyme, a reasonable assumption is that it may be related to the control mechanism. Two relatively simple mechanisms are quantitatively consistent with the data. One mechanism postulates a rapid bimolecular reaction followed by a relatively slow isomerization of the complex formed:

$$E + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EM \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EM' \qquad (3)$$

where EM′ is the isomerized complex. This mechanism assumes that the binding sites are all independent and equivalent. The slowest relaxation time can be expressed as

$$1/\tau = k_{-2} + \frac{k_2}{1 + k_{-1}/\{k_1[(\bar{E}) + (\bar{M})]\}} \qquad (4)$$

and the data are quantitatively described by this equation.

A second mechanism consistent with the data is the MWC model. For a six-subunit enzyme this mechanism can be written as

$$\begin{array}{ccc} 6M + R_0 & \rightleftarrows & T_0 + 6M \\ \Updownarrow & & \Updownarrow \\ 5M + R_1 & \rightleftarrows & T_1 + 5M \\ \Updownarrow & & \Updownarrow \\ 4M + R_2 & \rightleftarrows & T_2 + 4M \\ \Updownarrow & & \Updownarrow \\ 3M + R_3 & \rightleftarrows & T_3 + 3M \\ \Updownarrow & & \Updownarrow \\ 2M + R_4 & \rightleftarrows & T_4 + 2M \\ \Updownarrow & & \Updownarrow \\ M + R_5 & \rightleftarrows & T_5 + M \\ \Updownarrow & & \Updownarrow \\ R_6 & \rightleftarrows & T_6 \end{array} \qquad (5)$$

where R and T are different conformational states of the enzyme.

According to this model, in the absence of ligand, the enzyme exists mainly in the T state so that the interconversion of R and T states cannot be observed. With excess amounts of carbamyl phosphate and succinate, however, the enzyme has converted largely to the R states, and addition of BrCTP tends to cause the conversion from R to T states so that the conformational changes can be observed under these conditions. In this case, the rate of interconversion of the two conformational states of the enzyme increases as BrCTP is added. The experimental data are also quantitatively consistent with this mechanism (*17*). Thus for BrCTP binding in the presence of substrates, either the MWC model or the very simple mechanism of Eq. 3 is sufficient to account for the data.

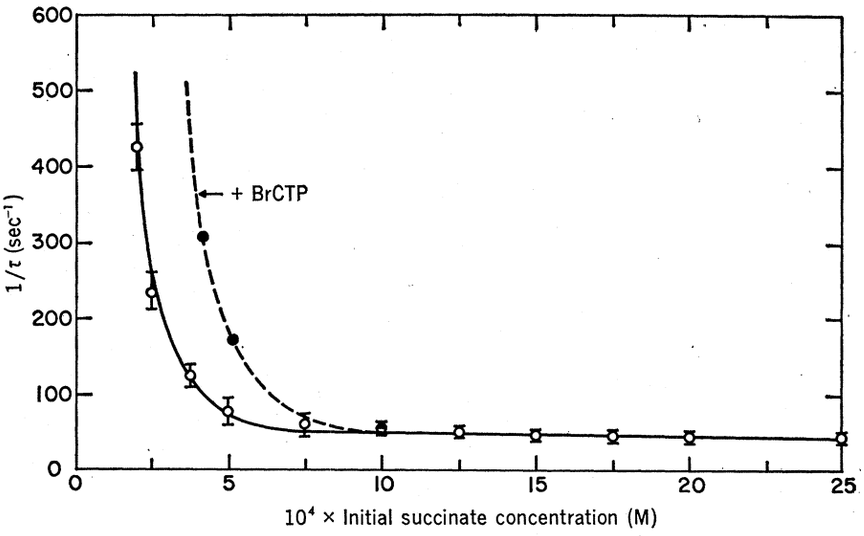


Fig. 5. Plot of the reciprocal relaxation time, $1/\tau$, versus the initial concentration of succinate in the presence of 1 m*M* carbamyl phosphate. The solid line has been calculated on the assumption that the MWC model is valid; the dashed line indicates the effect on the reciprocal relaxation time of the addition of 0.25 m*M* BrCTP to the system.

If the MWC model is correct, then similar phenomena should be observed for the binding of substrates or substrate analogs. Although only small difference spectra are produced by the binding of carbamyl phosphate and L-aspartate or aspartate analogs such as L-malate or succinate, the kinetics of the binding processes can be studied by observing the *p*H changes accompanying binding with *p*H indicators. In the absence of carbamyl phosphate, no relaxation process was detected in solutions containing L-aspartate analogs and native aspartate transcarbamylase or its catalytic subunit. In the presence of saturating concentrations of carbamyl phosphate, two relaxation processes are associated with the binding of succinate to the complex of catalytic subunit and carbamyl phosphate (*18*). These fall in the time ranges of 100 to 500 and 20 to 50 microseconds, and can be quantitatively analyzed in terms of a two-step mechanism with a rapid bimolecular reaction followed by a relatively slow isomerization or conformational change (Eq. 3, where M is now the substrate). Similar studies with L-malate, a substrate analog with a hydroxyl group in the position of the α-amino group of L-aspartate, revealed a single relaxation process ($\tau \sim 50$ to 100 μsec) that could be associated with an isomerization of the enzyme-substrate complex according to Eq. 3, although the kinetics of the bimolecular step could not be studied. The rate constant characterizing the conversion of the second enzyme-substrate complex to the first complex is ten times larger for L-malate, although the rate constants for the reverse processes are quite similar in both cases. This suggests that the α substituent in the L form encounters steric interference in the isomerized complex, a condition consistent with a mechanism whereby catalysis occurs through a conformational change that forces the substrates together (*21*).

Quite different relaxation processes were observed for succinate and L-malate binding to the native enzyme (*19*). A single relaxation process, much slower ($\tau \sim 1$ to 20 msec) than that observed with the catalytic subunit, was found to accompany both succinate and L-malate binding. The relaxation times are independent of the enzyme concentration. Moreover, the dependence of the relaxation time on the concentration of L-aspartate analogs is rather unusual: the reciprocal relaxation time decreases as the concentration of succinate or L-malate increases and approaches a constant value at high concentrations, as shown in Fig. 5. A simple mechanism consistent with this behavior is a mechanism such as the MWC model. In these cases, the rate of interconversion of the two enzyme conformations (R and T states) decreases as the amount of substrate analog bound increases, and, in fact, the

data are quantitatively consistent with the MWC model (Eq. 5). Since an analogous process is not observed with the catalytic subunit, it is probably involved in the allosteric control mechanism. The processes observed in the catalytic subunit also occur in the native enzyme but, as will be indicated below, require somewhat different conditions for detection.

A surprising result was obtained when both BrCTP and succinate were present with saturating carbamyl phosphate. Two relaxation processes could be observed which obviously corresponded to the processes associated with the conformational transitions of enzyme induced by BrCTP or succinate alone as described above. Furthermore, the value of the relaxation time associated with succinate binding is dependent on the BrCTP concentration. In fact, the effect of BrCTP is to shift the curve of Fig. 5 to the right on the concentration axis, as shown. This is quite analogous to the change in the aspartate saturation curve caused by CTP (Fig. 2). The relaxation process associated with BrCTP binding also varies with succinate concentration. These synergistic effects between substrate and effector support the contention that regulation processes are being observed.

The above results are inconsistent with the simple two-state MWC model. This model postulates that control is exerted by shifting the equilibrium between two conformations of the enzyme. If this were correct, only one relaxation process associated with regulation would be observed in the presence of both succinate and BrCTP. In other words, if only two conformations exist, but the mechanism of interconversion is different when BrCTP or succinate is bound, then, to a good approximation, only the faster mechanism would occur when both BrCTP and succinate are present. Instead, two coupled, but distinct, conformational changes are observed. A simple schematic model consistent with the above results is

$$\begin{array}{ccc} \mathrm{Rr} & \rightleftarrows & \mathrm{Tr} \\ \Updownarrow & & \Updownarrow \\ \mathrm{Rt} & \rightleftarrows & \mathrm{Tt} \end{array} \qquad (6)$$

Four different conformational states are postulated here. The horizontal T→ R transitions are induced by aspartate analogs in the presence of saturating carbamyl phosphate, whereas the vertical r → t transitions are induced by BrCTP in the presence of carbamyl phosphate and succinate. Both the R-T or r-t transitions are in accord with the MWC model, although the r-t transition might involve the alternative mechanism discussed earlier. The Rr species binds substrate the best, and the Tt species binds substrate the worst. Ligands that shift the conformational equilibriums from R to T or r to t inhibit enzyme activity, and those shifting the equilibriums in the opposite direction activate the enzyme. The above model is sufficient to account for all of the results discussed thus far. However, even this relatively complex scheme fails to account for the results obtained when the concentration of carbamyl phosphate is varied (*18, 20*).

In the absence of aspartate analogs, a single relaxation process ($\tau \sim 0.1$ to 0.5 msec) is observed to accompany the binding of carbamyl phosphate to both the native enzyme and the catalytic subunit. The simple bimolecular mechanism of Eq. 1 is consistent with the data, and, in fact, identical equilibrium dissociation constants are obtained in both cases ($4 \times 10^{-4} M$). No evidence of conformational changes related to the control process could be detected.

In the presence of 10 mM succinate, the situation becomes much more complex. Two relaxation processes could be associated with the binding of carbamyl phosphate to the native enzyme. The faster process ($\tau \sim 0.2$ to 1 msec) occurs in a time range similar to that observed in the absence of succinate, but the concentration dependence is quite different. The reciprocal relaxation time increases linearly with the sum of the equilibrium concentrations of enzyme and substrate initially, but eventually plateaus at high concentrations. The data are quantitatively consistent with the two-step mechanism of Eq. 2 if the assumption is made that the two steps are kinetically coupled. Qualitatively the bimolecular step is rate limiting at low concentrations, but the conformational change becomes rate limiting at high concentrations. An exact solution of the kinetic problem quantitatively fits the data over the entire concentration range. Surprisingly, the rate constants associated with the conformational change are quite similar to those associated with the conformational change accompanying succinate binding to the complex of catalytic subunit and carbamyl phosphate, which is probably of importance in the catalytic mechanism. In fact, these two conformational changes are very likely the same process. The actual mechanism of binding to the native enzyme should be written as

$$\mathrm{E} + \mathrm{CP} \rightleftarrows \mathrm{ECP} \underset{\text{fast}}{\overset{\mathrm{S}}{\rightleftharpoons}} \mathrm{ECPS} \rightleftarrows \mathrm{ECPS}' \qquad (7)$$

where CP and S are carbamyl phosphate and succinate, ECP is the enzyme–carbamyl phosphate complex, and ECPS and ECPS′ are two different conformations of the enzyme–carbamyl phosphate–succinate complex. In the absence of succinate, the conformational change does not take place. On the other hand, the second step equilibrates rapidly with saturating amounts of succinate so that the overall effect observed will be the coupling of steps 1 and 3, which is kinetically indistinguishable from the mechanism given in Eq. 3. This ordered binding mechanism (Eq. 7), with succinate binding after carbamyl phosphate, has been suggested by numerous other results (*12, 15, 19*). Furthermore, the proposition that this conformational change is associated with the catalytic mechanism, rather than with the regulatory mechanism, is supported by the finding that the addition of BrCTP to the system has no observable effect on the relaxation time associated with this process.

The slower relaxation process ($\tau \sim$ 25 to 50 msec) has a concentration dependence which is not consistent with the binding mechanism of Eqs. 1 or 3; it increases with increasing concentration of carbamyl phosphate, but not in a simple manner. However, the MWC model (Eq. 5) is quantitatively consistent with the data. This process may be related to the slow process that accompanies succinate binding to the native enzyme–carbamyl phosphate complex. Nevertheless, a number of observations suggest that these two processes may not be the same. The reciprocal relaxation time increases with increasing carbamyl phosphate concentration but decreases with increasing succinate concentration; also BrCTP decreases the reciprocal relaxation time for the process associated with carbamyl phosphate binding and increases it for that accompanying succinate binding. Since BrCTP alters the value of this slow relaxation time and the conformational change is observed only with the native enzyme, it can be presumed to be of significance in the control process. Thus, at least three different conformational

changes can be associated with the control mechanism. All of these display some synergistic effect between BrCTP and succinate and carbamyl phosphate binding.

Conclusions

Although a number of questions remain to be answered, the mechanism of the control process for aspartate transcarbamylase has been broken down into several discrete steps. The conclusions which can be derived from these results (see Table 1) may be summarized as follows. The bimolecular reactions of BrCTP and of carbamyl phosphate with the enzyme are quite similar for the native enzyme and its subunits, and antagonistic effects between effectors and substrates are not observed. Also one of the conformational changes induced by succinate and carbamyl phosphate is essentially the same for the native enzyme and catalytic subunit. Since BrCTP has no effect on this process, it is presumably part of the catalytic mechanism. Finally, three distinct conformational changes are seen only with the native enzyme in the presence of various combinations of carbamyl phosphate, succinate, and BrCTP. Moreover, the rates of the conformational changes display a synergistic relation between BrCTP, carbamyl phosphate, and succinate. This implies that these conformational changes are involved in the control mechanism.

The conformational changes associated with carbamyl phosphate and aspartate-analog binding appear to be concerted in nature, and that associated with BrCTP may be concerted or may involve a stepwise mechanism. A simple sequential mechanism which predicts a spectrum of relaxation processes is not consistent with the experimental data, although a more complex sequential mechanism cannot be ruled out. In any event, the overall control mechanism is apparently a combination of several molecular changes and must be considerably more complex than the limiting models discussed earlier. The results obtained indicate that a number of different conformational transitions can lead to the same end result: enhancement or inhibition of the enzyme activity. This multiplicity of conformational changes involved in the regulatory mechanism is a desirable and reasonable feature: it provides versatility and sensitivity of the control mechanism to a variety of different molecules.

Finally mention should be made of the fact that the approach used in this particular case, that is, resolution of the elementary steps on the time axis, is of general utility both in the study of enzymatic catalysis (*22*) and of control processes for other enzymes, such as yeast glyceraldehyde-3-phosphate dehydrogenase (*23*), homoserine dehydrogenase (*24*), and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (*25*). In fact, fast reaction techniques are quite general and powerful techniques for the elucidation of the molecular details of biochemical mechanisms.

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