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Diagnostic Relationships in the Relaxation Spectrometry of Allosteric Enzymes*

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ABSTRACT: Because the relaxation spectra of multisubunit proteins showing cooperative effects are very complex, a systematic study of alternative binding patterns for a dimeric enzyme was made to determine diagnostic relationships between relaxation spectra and mechanism of ligand binding. These diagnostic relationships reveal patterns which can be generalized to larger systems. The relaxation rates show a range of concentration dependencies which may increase indefinitely, decrease to an asymptote, reach a maximum, etc. Different models for ligand binding will often predict relaxation spectra which are kinetically indistinguishable. A consideration of the experimental problems involved reveal that the shapes of the concentration dependence of relaxation rate curves can be used diagnostically, but that the mechanisms so deduced must be considered to be working hypotheses rather than final conclusions because of the assumptions required to solve the complex mathematics.

In understanding the nature of allosteric regulation in metabolic systems it is important to relate the structure of the protein to the kinetic phenomena associated with the enzyme. Various techniques are being employed to correlate kinetics with information about structure and function of enzymes, but one of the most important methods is the use of relaxation spectrometry, particularly the temperature-jump method. Binding data and steady-state kinetics can provide very important information, but many steps of an enzymatic reaction proceed at velocities which exceed those accessible to these techniques.

The application of the temperature-jump method to the study of very fast reactions in allosteric proteins has been pioneered by Eigen (1954) and his coworkers and applied to the specific protein yeast glyceraldehyde-3-phosphate dehydrogenase (Kirschner *et al.*, 1966). The details of the method are well documented in the classic treatise of Eigen and de Maeyer (1963), and reviews of the subject in varying degrees of mathematical complexity have been published (Czerlinski, 1967; Hammes, 1968, 1969; Faller, 1969; French and Hammes, 1969; Gutfreund, 1971).

To prove a mechanism it is not simply acceptable to show that the data are consistent with that mechanism, but one must also show that the data are incompatible with reasonable alternative mechanisms. Because kinetic derivations of relaxation spectrometry beyond the simplest systems are complex, most fast reaction papers have reported data consistent with one mechanism and occasionally discuss one alternative which does not fit the data. Practically never is the potential range of consistent mechanisms discussed, yet the scientist interested in protein properties wishes to know in a given situation what mechanisms have been "proved" or "ex-

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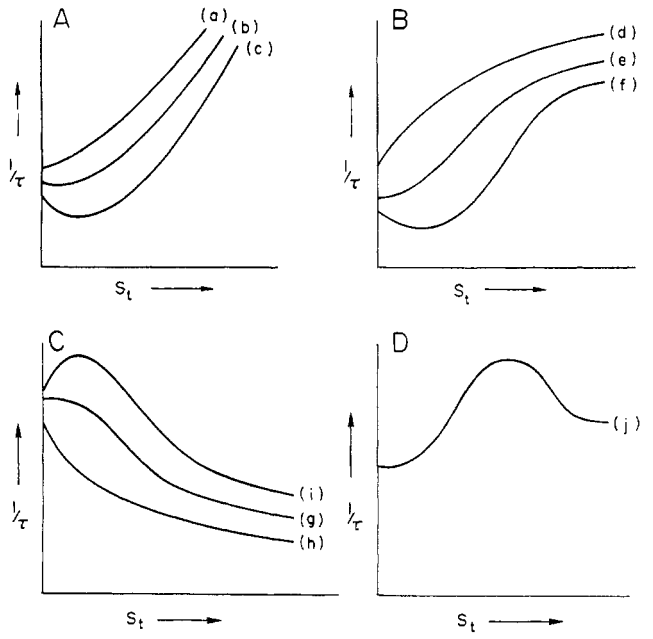


FIGURE 1: Qualitative nature of curves observed for various fast relaxation processes. E_T is held constant. Letters a, b...j identify curves referred to in Table II.

cluded" and how definitive is temperature-jump data relative to other techniques such as equilibrium dialysis or the use of spin labels. It seemed important therefore to take the simplest cooperative system, a dimer or two site model, and systematically consider a wide range of alternatives suggested by current models and experimental findings (Monod *et al.*, 1965; Koshland *et al.*, 1966; Koshland, 1970; Atkinson, 1970; Stadtman, 1970).

In the process of deriving equations it rapidly became evident that even a system as simple as a dimer cannot be resolved rigorously. Assumptions must be made in regard to the number of measurable steps and the separation of the relaxation times in order to make any progress in solving the equations. However, it is possible to maintain a reasonably consistent set of assumptions and consider systematically the various alternatives for a dimer. This has been done and in the process a few generalizations applicable to more complex systems have emerged.

Glossary

In the discussions which follow we shall introduce certain notations in order to simplify the mathematical expressions and to make the individual rate constants in different mechanisms easily recognizable. In general, we shall use k to represent rate constants and K to represent equilibrium constants. We shall use β_i and γ_i to represent the ith binding step, and the ith conformational step, respectively. When indicating rate constants, we shall use $k_{\beta i}$ to indicate the forward reaction and $k_{i\beta}$ to indicate the reverse reaction in a binding step, and a similar notation for the conformational rate constants $k_{\gamma i}$ and $k_{i\gamma}$. This is illustrated for a simple process in eq 1. If both bindings and a conformational change occur in a single step, then the notation $(\beta\gamma)_i$ will be used in analogy with the previous definition. Equilibrium constants will be denoted as $K_{\gamma i}$ or $K_{i\gamma}$, which refer to the ratios $k_{\gamma i}/k_{i\gamma}$ and $k_{i\gamma}/k_{\gamma i}$, respectively, and $K_{\beta i}$ or $K_{i\beta}$, which refer to the ratios $k_{\beta i}/k_{i\beta}$ and $k_{i\beta}/k_{\beta i}$, respectively (*cf.* eq 1).

$$\mathrm{S} + \bigcirc \underset{k_{1\beta}}{\overset{k_{\beta 1}}{\rightleftharpoons}} \textcircled{S} \underset{k_{1\gamma}}{\overset{k_{\gamma 1}}{\rightleftharpoons}} \boxed{\mathrm{S}} \qquad K_{1\beta} = \frac{k_{1\beta}}{k_{\beta 1}} \quad K_{\gamma 1} = \frac{k_{\gamma 1}}{k_{1\gamma}} \tag{1}$$

In discussing mechanisms in this article we shall find it convenient to provide a shorthand which indicates the relative magnitudes of the various relaxation rates in a multistep scheme, since such information automatically indicates which steps are mutually coupled. Although the subscript i gives the number of the step, it will also be convenient to attach a prime to constants or symbols associated with the faster of two processes. This notation can be generalized to n steps. The constants or symbols associated with the most rapid step(s) will have $n - 1$ primes, those corresponding to the next most rapid step will have $n - 2$ primes, and so on, with the symbols associated with the slowest step having no primes. If two or more steps in a scheme have relaxation rates which are of the same order of magnitude, *i.e.*, they cannot be separated in analysis, their symbols will contain the same prime designation. Hence, in considering a binding scheme for a two-subunit enzyme in which conformational changes follow binding in each subunit, let us assume that the relaxation rates are in the order

$$1/\tau_{\beta 1} \sim 1/\tau_{\beta 2} \gg 1/\tau_{\gamma 1} \gg 1/\tau_{\gamma 2} \tag{2}$$

Thus, the scheme would be abbreviated $\beta_1''\gamma_1'\beta_2''\gamma_2$. Since relaxation rates are functions of concentration, this notation only makes sense, and will only be used, when two or more observable relaxation rates are well separated in time over the accessible concentration range or when we are speaking of relaxation rates at a particular concentration. This special case will assume considerable importance in the discussions to follow.

The symbols $\bar{S}$, $\bar{E}$, S_t, and E_t will also be used to designate the equilibrium substrate, the equilibrium enzyme, the total substrate, and total enzyme concentrations, respectively.

Theoretical

Case 1. The simplest case of a conformational change in a binding scheme, in which a ligand-induced conformational change occurs simultaneously on binding of a substrate, is shown in eq 3. The reciprocal relaxation time, $1/\tau$, for

$$\mathrm{S} + \bigcirc \underset{k_{i,\beta\gamma}}{\overset{k_{\beta\gamma,i}}{\rightleftharpoons}} \boxed{\mathrm{S}} \tag{3}$$

$$1/\tau = k_{\beta\gamma,1}(\bar{E} + \bar{S}) + k_{1,\beta\gamma} \tag{4}$$

this process is given in eq 4 (Eigen and de Maeyer, 1963). This equation is normally plotted as $1/\tau$ *vs.* $(\bar{E} + \bar{S})$ to give a straight line whose slope or intercept yields the desired rate constants of the system. For comparison with cases to be presented later one may also plot $1/\tau$ *vs.* S_t, where S_t is the total substrate concentration, which will give the curves a, b, and c of Figure 1A. The important feature of all of these curves is that the reciprocal relaxation time increases without limit as S_t increases.

Case 2. A slightly more complex case is that of eq 1 in which a binding step is followed by a conformational change. The mathematics of such a simple mechanism can be relatively complex, but in the case designated $\beta_1\gamma_1'$ in which there is a slow binding step followed by a very rapid conformational change the mathematics can be solved very easily. Using approximation methods (Hammes, 1968) the observable reciprocal relaxation time is given by eq 4a. It will give plots

$$1/\tau_{\beta 1} = k_{\beta 1}(\bar{E} + \bar{S}) + \frac{k_{1\beta}}{k_{\gamma 1}' + 1} \tag{4a}$$

similar to those of curves a, b, and c of Figure 1A in that they increase without limit as $S_t \rightarrow \infty$.

If the binding is followed by a conformational isomerization which is not very rapid, but which has a relaxation rate of the same order of magnitude as that of the binding, the kinetic situation is more complex. The mathematics of an essentially identical case have been worked out by Eigen and de Maeyer (1963). The relaxation rates for both processes are given, respectively, by the alternate solutions of quadratic eq 5 where $\alpha = k_{\beta 1}(\bar{E} + \bar{S}) + k_{1\beta} + k_{\gamma 1} + k_{1\gamma}$ and $\omega =$

$$1/\tau = {}^1/_2[\alpha \pm \sqrt{\alpha^2 + 4\omega}] \tag{5}$$

$k_{\beta 1}\cdot(\bar{E} + \bar{S})(k_{\gamma 1} + k_{1\gamma}) + k_{1\beta}k_{1\gamma}$. For a two-subunit enzyme involving a binding and conformational process in each subunit (four relaxations), the corresponding analytical solution could be derived by solving a secular equation of the fourth order, and enzymes of larger size would increase exponentially in complexity. Thus, even the simplest calculations for allosteric proteins are considerably more complex than eq 1 and cannot be solved without approximations or very complicated solutions.

Solutions with Simplifying Assumptions

In order to evaluate what types of curves might be diagnostic for a given mechanism, simplifying cases have been analyzed and compared. One simplifying assumption is that all measurable relaxation rates are sufficiently different that they may be analyzed separately. A difference of a factor of 100 in relaxation rates will make the approximations shown below, which use this assumption, excellent, and relaxation rates which approach each other more closely than this will make the use of the approximations correspondingly less accurate. Since the relaxation rates are functions of concentration, two or more relaxation rates which differ little over one range of concentration may differ considerably over another. Thus, these approximations may be made valid in many cases by adjustment of the experimental conditions. It is the relative magnitudes of the $1/\tau$ values which determine the "separability" of two processes in analysis (Eigen, 1968). This does not mean that the approximations which we shall use apply to every enzyme, but just as we assume in ordinary kinetics that there are rapid, reversible binding steps followed by rate-determining steps, these approximations lead to predictions which can be tested to demonstrate their validity.

Applying this procedure to the mechanism of eq 1 we can assume as a first example that the binding step reequilibrates much faster than the conformational change step, *i.e.*, a mechanism designated as $\beta_1'\gamma_1$. In this case the relaxation rate identified with the binding step will occur essentially independently of the second step and will have a concentration dependence given in eq 6. The plot of this dependence will be

$$1/\tau_{\beta 1}' = k_{\beta 1}'(\bar{E} + \bar{S}) + k_{1\beta}' \tag{6}$$

similar to those of curves a, b, and c of Figure 1A. The second relaxation occurs more slowly than the first and hence is coupled to the first. The solution for the second relaxation rate is given by eq 7 as deduced by Hammes (1969). Equation 7 has

$$1/\tau_{\gamma 1} = k_{\gamma 1}\left[\frac{\bar{E} + \bar{S}}{K_{1\beta}' + \bar{E} + \bar{S}}\right] + k_{1\gamma} \tag{7}$$

arisen from the interpretation of a number of relaxation phenomena with simple enzymes (Holler *et al.*, 1968; Hess, 1968) and a plot of $1/\tau_{\gamma 1}$ *vs.* $(\bar{E} + \bar{S})$ gives a simple hyperbolic curve.

For purposes of analogy with more complex cases to be presented later, we would like to be able to express relaxation rates not as functions of $\bar{E}$ and $\bar{S}$, which require special independent equilibrium measurements, but rather as a function of the easily determined variables E_t and S_t. This change of variables would enable the experimentalist to rapidly plot his raw data and obtain a preliminary mechanistic diagnosis without having to resort to more involved experiments or calculations necessary to obtain $\bar{E}$ and $\bar{S}$, or the concentrations of other intermediate enzyme forms. Such a change of variable, while perfectly feasible, once again results in higher order polynomial equations even for the simplest mechanisms. This problem can be avoided when we are interested in gaining some qualitative insights into the concentration dependence of a given relaxation if we express $\bar{E}$ in terms of E_t using the simple mass balance relationships of eq 8 and 9, in which, for

$$\bar{E} = E_t\nu \tag{8}$$

$$\nu = \frac{K_{1\beta}'}{K_{1\beta}' + \bar{S}} \tag{9}$$

example, for a simple binding, as in eq 2, ν is given by eq 9. This same device can be used for any mechanism, and ν will be a function of $\bar{S}$ characteristic of the mechanism. Substituting eq 8 into eq 7, we obtain eq 10. We are now in a position

$$1/\tau_{\gamma 1} = k_{\gamma 1}\left[\frac{E_t\nu + \bar{S}}{K_{1\beta}' + E_t\nu + \bar{S}}\right] + k_{1\gamma} \tag{10}$$

to obtain information about the dependence of $1/\tau_{\gamma 1}$ on S_t from the limiting values of the reciprocal relaxation time at small and large S_t, respectively. As S_t approaches 0, ν approaches 1, and the expression for the reciprocal relaxation time is given in eq 11. As S_t approaches very high values (indicated by "$S_t \rightarrow \infty$"), then $\bar{S} \rightarrow S_t$, and only highest order terms of $\bar{S}$ dominate the expression. Equation 12 gives the

$$\lim_{S_t \rightarrow \infty} 1/\tau_{\gamma 1} = k\frac{E_t}{K_{1\beta}' + E_t} + k_{1\gamma} \tag{11}$$

$$\lim_{S_t \rightarrow \infty} 1/\tau_{\gamma 1} = k_{\gamma 1} + k_{1\gamma} \tag{12}$$

limiting relaxation rate. Equation 12 is identical with the expression obtained for the relaxation rate of a unimolecular isomerization not coupled to any other step.

TABLE I. Concentration Dependence of Relaxation Rates for Case 4: ($\beta_1\ \gamma_1\ \beta_2\ \gamma_2$)

A. ($\beta_1''\ \gamma_1\ \beta_2''\ \gamma_2'$)

(1) Consideration of γ_1:

$$1/\tau_{\gamma 1} = \frac{2k_{\gamma 1}\left\{[K'_{\gamma 2}+1]\left[(\bar{E})\left(\frac{K_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}} + \frac{\bar{S}}{K''_{1\beta}}\right) + \frac{\bar{S}^2}{2K''_{1\beta}}\right] + \bar{E} + \bar{S}\right\} + 2k_{1\gamma}\left\{[K'_{\gamma 2}+1][\bar{E}]\left[\frac{K_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}} + \frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}}\right] + \bar{E} + \bar{S} + K_{1\beta}\right\}}{[K'_{\gamma 2}+1]\left[(\bar{E})\left(\frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}} + \frac{\bar{S}}{K''_{1\beta}} + \frac{K_{\gamma 1}\bar{S}^2}{K''^2_{1\beta}}\right) + \bar{S} + \frac{\bar{S}^2}{K''_{1\beta}}\right] + 2(\bar{E}+\bar{S})}$$

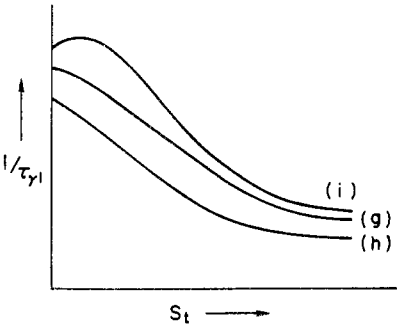
Limits:

$\lim_{S_t \to 0} 1/\tau_{\gamma 1} = k_{\gamma 1} + k_{1\gamma}\left[\frac{E_t + K''_{1\beta}}{E_t}\right]$

$\lim_{S_t \to \infty} 1/\tau_{\gamma 1} = k_{\gamma 1}$

$\lim_{S_t \to 0} \frac{d(1/\tau_{\gamma 1})}{dS_t}$ may be >0, <0, or $=0$

$\lim_{S_t \to \infty} \frac{d(1/\tau_{\gamma 1})}{dS_t} = 0$



(2) Consideration of γ_2':

$$1/\tau'_{\gamma 2} = \frac{k'_{\gamma 2}\left[(\bar{E})\left(\frac{\bar{S}}{K''_{1\beta}} + \frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}} + \frac{K_{\gamma 1}\bar{S}^2}{K''^2_{1\beta}}\right) + \bar{S} + \frac{\bar{S}^2}{2K''_{1\beta}}\right] + k'_{2\gamma}\left[(\bar{E})\left(\frac{\bar{S}}{K''_{1\beta}} + \frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}} + \frac{K_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}} + 2\right) + 3\bar{S} + \frac{\bar{S}^2}{2K''_{1\beta}}\right]}{(\bar{E})\left(\frac{\bar{S}}{K''_{1\beta}} + \frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}} + \frac{K_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}} + 2\right) + 3\bar{S} + \frac{\bar{S}^2}{2K''_{1\beta}}}$$

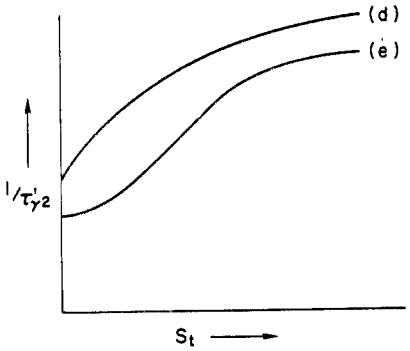
Limits:

$\lim_{S_t \to 0} 1/\tau'_{\gamma 2} = k'_{2\gamma}$

$\lim_{S_t \to \infty} 1/\tau'_{\gamma 2} = k'_{\gamma 2} + k'_{2\gamma}$

$\lim_{S_t \to 0} \frac{d(1/\tau'_{\gamma 2})}{dS_t} \geq 0$ only

$\lim_{S_t \to \infty} \frac{d(1/\tau'_{\gamma 2})}{dS_t} = 0$



To compute the limiting slopes it is most simple to perform calculations by obtaining the derivative as shown in eq 13

$$\frac{d(1/\tau_{\gamma 1})}{dS_t} = \frac{d(1/\tau_{\gamma 1})}{d\bar{S}}\frac{d\bar{S}}{dS_t} \tag{13}$$

and then taking the limiting value of this derivative at large and small S_t. This information, together with eq 11 and 12, enable us to describe the general concentration dependence of $1/\tau_{\gamma 1}$. This is shown in Figure 1B.

Case 3. A conformational change followed by a binding step ($\gamma_1\beta_1$) is shown in eq 14. If we now assume that the binding step is the more rapid of the two processes and the conformational step is slow ($\gamma_1\beta_1'$), the binding relaxation rate may be treated independently and is given by eq 15, in which $\bar{E}$ represents the "circle form" of the free enzyme. This relaxation rate would have the concentration dependence shown in

$$K_{\gamma 1} = \frac{k_{\gamma 1}}{k_{1\gamma}}$$

$$\bigcirc \underset{k_{1\gamma}}{\overset{k_{\gamma 1}}{\rightleftharpoons}} \square \underset{k_{1\beta}}{\overset{k_{\beta 1}(S)}{\rightleftharpoons}} \boxed{S} \tag{14}$$

$$K_{1\beta} = \frac{k_{1\beta}}{k_{\beta 1}}$$

$$1/\tau_{\beta 1}' = k_{\beta 1}'(K_{\gamma 1}\bar{E} + \bar{S}) + k_{1\beta}' \tag{15}$$

Figure 1A. The conformational relaxation rate will then be given by eq 16 and the limiting values of $1/\tau_{\gamma 1}$ and its derivative by eq 16a,b. This information allows us to construct the plots shown in Figure 1C. This reciprocal relaxation time is

$$1/\tau_{\gamma 1} = k_{\gamma 1} + k_{1\gamma}\left[\frac{K_{1\beta}' + K_{\gamma 1}\bar{E}}{K_{1\beta}' + K_{\gamma 1}\bar{E} + \bar{S}}\right] \tag{16}$$

TABLE I, Contd.

B. $(\beta_1''\gamma_1\ \beta_2''\gamma_2)$

(1) Consideration of γ_2:

$$1/\tau_{\gamma 2} = \frac{k_{\gamma 2}\left\{[\bar{E}]\left[4K'_{\gamma 1}\bar{S} + (K'_{\gamma 1}+1)\frac{K'_{\gamma 1}\bar{S}^2}{K''_{1\beta}}\right] + K_{\gamma 1}\bar{S}^2\right\}}{[\bar{E}]\left[(K'_{\gamma 1}+1)\left(2K''_{1\beta} + \frac{K'_{\gamma 1}\bar{S}^2}{K''_{1\beta}}\right) + 4K'_{\gamma 1}\bar{S}\right] + 2K''^{2}_{1\beta} + 2K''_{1\beta}(K'_{\gamma 1}+1)\bar{S} + K'_{\gamma 1}\bar{S}^2} + k_{2\gamma}$$

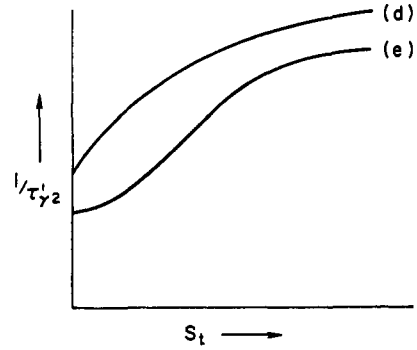
Limits:

$\lim_{S_t\to 0} 1/\tau_{\gamma 2} = k_{2\gamma}$

$\lim_{S_t\to \infty} 1/\tau_{\gamma 2} = k_{\gamma 2} + k_{2\gamma}$

$\lim_{S_t\to 0} \frac{d(1/\tau_{\gamma 2})}{dS_t} \geq 0$ always

$\lim_{S_t\to \infty} \frac{d(1/\tau_{\gamma 2})}{dS_t} = 0$



(2) Consideration of γ_1':

$$1/\tau'_{\gamma 1} = \frac{2k'_{\gamma 1}\left[(\bar{E})\left(2\bar{S} + \frac{K'_{\gamma 1}\bar{S}}{K''_{1\beta}} + 2K''_{1\beta}\bar{S} + \bar{S}^2\right)\right] + 4k'_{1\gamma}K''_{1\beta}\left[(\bar{E})\left(1 + \frac{K'_{\gamma 1}\bar{S}}{K''_{1\beta}} + \frac{K'_{\gamma 1}\bar{S}^2}{2K''^{2}_{1\beta}}\right) + \bar{S} + K''_{1\beta}\right]}{(\bar{E})\left(2K'_{\gamma 1}\bar{S} + \frac{\bar{S}}{K''_{1\beta}} + \frac{K'_{\gamma 1}\bar{S}^2}{K''^{2}_{1\beta}} + 4K''_{1\beta}\right) + 6K''_{1\beta}\bar{S} + 2\bar{S}^2 + 4K''^{2}_{1\beta}\bar{S}}$$

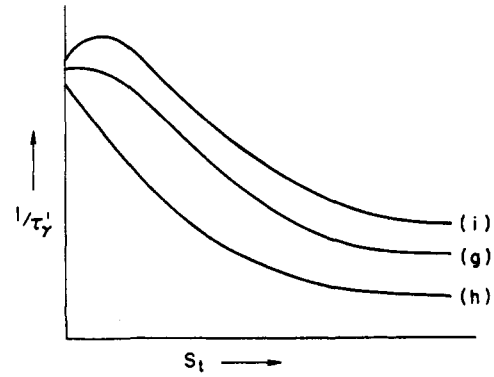
Limits:

$\lim_{S_t\to 0} 1/\tau'_{\gamma 1} = k'_{\gamma 1} + k'_{1\gamma}\left[\frac{E_t + K''_{1\beta}}{E_t}\right]$

$\lim_{S_t\to \infty} 1/\tau'_{\gamma 1} = k'_{\gamma 1}$

$\lim_{S_t\to 0} \frac{d(1/\tau'_{\gamma 1})}{dS_t}$ may be >0, <0, or $=0$

$\lim_{S_t\to \infty} \frac{d(1/\tau'_{\gamma 1})}{dS_t} = 0$

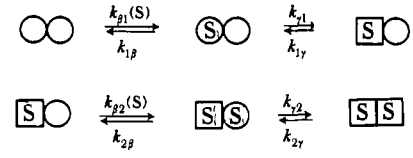


$$\lim_{S_t\to 0}(1/\tau_{\gamma 1}) = k_{\gamma 1} + k_{1\gamma} \qquad \lim_{S_t\to \infty}(1/\tau_{\gamma 1}) = k_{\gamma 1} \quad (16a)$$

$$\lim_{S_t\to 0}\frac{d(1/\tau_{\gamma 1})}{dS_t} \leqq 0 \qquad \lim_{S_t\to \infty}\frac{d(1/\tau_{\gamma 1})}{dS_t} = 0 \quad (16b)$$

observed to decrease between the two limiting values of $1/\tau_{\gamma 1}$, namely, $(k_{\gamma 1} + k_{1\gamma})$, and $(k_{\gamma 1})$, with increasing S_t.

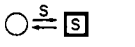
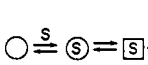
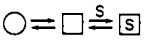
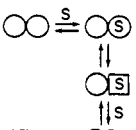
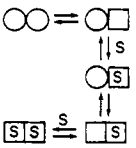
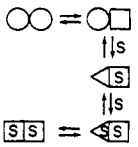
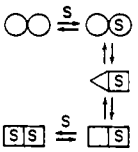
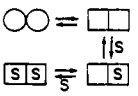
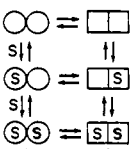
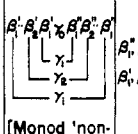
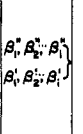
Case 4. If one considers a dimeric enzyme molecule in which subunit interactions occur, the number of permutations and combinations of conformational changes and bindings leading to a sequential saturation scheme increase appreciably. To simplify these situations and clarify the types of concentration dependence which might be expected for some typical mechanisms, we have extended the procedures outlined above for the monomeric cases to a dimeric enzyme. In this case, the binding of 2 moles of ligand to a two-subunit protein proceeds in steps according to eq 17, in which the binding process



(17)

precedes the conformational isomerization for each subunit. In this and all examples to follow in which binding and conformational steps occur as discreet processes, the binding relaxation rates will be assumed to be much faster than the conformational relaxations. Thus, the binding steps may be treated independently. Although they may differ from each other in detail, it is easy to show that the curves describing the relaxation rates of these binding steps in general will have

TABLE II Concentration Dependence of Relaxation Rates for Various Mechanisms of Ligand Binding to Monomeric or Dimeric Proteins

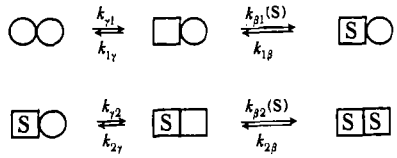
Case	Model	Abbreviation	Curves of $1/\tau$ vs. S_t Consistent with model		General diagnostic features of curves of $1/\tau$ vs. S_t consistent with model
			Process	Type of Curve*	
1		$(\beta_1\gamma_1)$	$(\beta_1\gamma_1)$	a,b,c	$1/\tau$ increases without limit as $S_t \to \infty$
2A		$\beta_1\gamma_1'$	β_1	a,b,c	$1/\tau$ increases without limit as $S_t \to \infty$
2B		$\beta_1'\gamma_1$	β_1'	a,b,c	$1/\tau$ increases without limit as $S_t \to \infty$
			γ_1	d,e,f	$1/\tau$ shows net increase to asymptotic limiting value; may show minimum
3		$\gamma_1\beta_1'$	β_1'	a,b,c	$1/\tau$ increases without limit as $S_t \to \infty$
			γ_1	g,h	$1/\tau$ shows net decrease to asymptotic limiting value
4A		$\beta_1''\gamma_1\beta_2''\gamma_2'$	γ_1	i,g,h	$1/\tau$ shows net decrease to asymptotic limiting value ; may show maximum
			γ_2'	d,e	$1/\tau$ shows net increase to asymptotic limiting value
4B		$\beta_1''\gamma_1'\beta_2''\gamma_2$	γ_2	d,e	$1/\tau$ shows net increase to asymptotic limiting value
			γ_1'	i,g,h	$1/\tau$ shows net decrease to asymptotic limiting value; may show maximum
5A		$\gamma_1\beta_1''\gamma_2'\beta_2''$	γ_1	g,h	$1/\tau$ shows net decrease to asymptotic limiting value
			γ_2'	d,j,f,i,g,h others possible	$1/\tau$ may increase, decrease, and go through maxima or minima; always approaches asymptotic limiting value
5B		$\gamma_1'\beta_1''\gamma_2\beta_2''$	γ_2	d,j,f,i,g,h others possible	$1/\tau$ may increase, decrease, and go through maxima or minima; always approaches asymptotic limiting value
			γ_1'	g,h	$1/\tau$ shows net decrease to asymptotic limiting value
6A		$\gamma_1\beta_1''\beta_2''\gamma_2'$	γ_1	g,h	$1/\tau$ shows net decrease to asymptotic limiting value
			γ_2'	d,e	$1/\tau$ shows net increase to asymptotic limiting value
6B		$\gamma_1'\beta_1''\beta_2''\gamma_2$	γ_2	d,e	$1/\tau$ shows net increase to asymptotic limiting value
			γ_1'	g,h	$1/\tau$ shows net decrease to asymptotic limiting value
7A		$\beta_1''\gamma_1\gamma_2'\beta_2''$	γ_1	d,j,f,i,g,h others possible	$1/\tau$ may increase, decrease, and go through maxima or minima; always approaches asymptotic limiting value
			γ_2'	g,h	$1/\tau$ shows net decrease to asymptotic limiting value
7B		$\beta_1''\gamma_1'\gamma_2\beta_2''$	γ_2	d,j,f,i,g,h others possible	$1/\tau$ may increase, decrease, and go through maxima or minima ; always approaches asymptotic limiting value
			γ_1'	d,e,f	$1/\tau$ shows net increase to asymptotic limiting value; may show minimum
8A		$(\gamma_1\gamma_2)\beta_1'\beta_2'$	$\beta_1\beta_2$	a,b,c	$1/\tau$ increases without limit as $S_t \to \infty$
		[Monod 'exclusive binding']	$(\gamma_1\gamma_2)$	g,h	$1/\tau$ shows net decrease to an asymptotic limiting value
8B				a,b,c	$1/\tau$ increases without limit as $S_t \to \infty$
		[Monod 'non-exclusive' binding']	$\gamma_1, \gamma_2 \cdots \gamma_i$	d,e,g,h	$1/\tau$ increases ($e > 1$) or decreases ($c < 1$) but reaches asymptotic limiting value at $S_t \to \infty$; shows no maxima or minima when the S-buffered assumption is used.

*Letters identify the curves, presented with a corresponding letter, in the Figures and Tables in the text.

the same diagnostic feature in common, namely, they increase without limit with increasing S_t. This type of dependence will also hold in subsequent cases in which the binding relaxations are assumed to be rapid. We shall not consider such rapid binding relaxations explicitly, and we need not differentiate which of the binding processes is faster as long as we recognize

that both are much faster than the conformational processes. We shall thus consider in order the two alternatives in which (a) the first of the two conformational steps is the slower, *i.e.*, the mechanism abbreviated $\beta_1''\gamma_1\beta_2''\gamma_2'$, and (b) the second of the two conformational steps is the slower, *i.e.*, $\beta_1''\gamma_1'\beta_2''\gamma_2$. It will also be assumed in this and subsequent cases that the conformational relaxation rates differ sufficiently that they may be separated in analysis. A detailed plot of the relaxation rate curves for such a model depends on the specific values of the rate constants, but important qualitative and quantitative features of the model can be obtained by analyzing limiting values of the reciprocal relaxation times and their slopes. The results for this case are summarized in Table I. Since the same qualitative curves are deduced from several mechanisms, we have used the device of designating the distinctive qualitative plots by the letters, a, b, c, etc., and indicating the curves consistent with the individual analytical expressions by the use of Table II and Figure 1. Thus in $\beta_2''\gamma_1\beta_2''\gamma_2'$ case of Table I, Table II shows that the analytical expression for $1/\tau_{\gamma 1}$ (case 4A) is compatible with curves i, g, and h of Figure 1. The curves compatible with case 4B are also referenced.

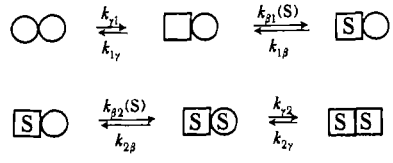
Case 5. Another potential pathway is one in which the conformational isomerizations precede the binding steps as shown in eq 18. Table III summarizes the relaxation rates which are



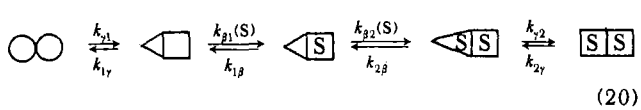
(18)

calculated for the two situations $\gamma_1\beta_1''\gamma_2'\beta_2''$ or $\gamma_1'\beta_1''\gamma_2\beta_2''$, and Tables II and Figure 1 show the plots resulting from a consideration of the limiting situations.

Case 6. A path in which the first isomerization occurs prior to binding and the second after binding is shown in eq 19,



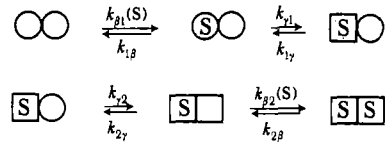
(19)



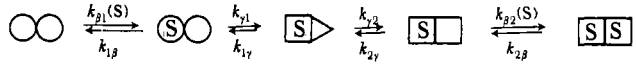
(20)

and would be abbreviated at $\gamma_1\beta_1\beta_2\gamma_2$. This does not seem to be a likely pathway, but a kinetically equivalent situation which is quite likely is shown in eq 20. In this case the binding of the first ligand partially alters the conformation of the adjacent subunit so that its affinity for the next molecule of ligand is increased. Following the second binding, another conformational change is induced which yields the final saturated enzyme species. The mathematical derivation holds equally well for pathways 19 and 20. The appropriate calculations and graphs are recorded in Tables II and III and Figure 1.

Case 7. In this case we consider the path of eq 21, abbreviated $\beta_1\gamma_1\gamma_2\beta_2$. A more realistic alternative which would give equivalent kinetics is shown in eq 22. In this case the first ligand binding causes the distortion of an adjacent subunit so that the conformational change must precede binding in the second step of the binding process. The calculations for this case are summarized in Tables II and III.



(21)



(22)

Other Cases. The mechanisms shown, of course, are not the only ones which would yield a sequential binding scheme. We showed under case 1 that if conformational changes were extremely rapid with respect to binding, the situation was kinetically indistinguishable from simple binding. In a multisubunit enzyme as well, one can envision a situation in which all conformation changes either occur much more rapidly than the accompanying binding steps or occur simultaneously with them. Such a scheme might be abbreviated $\beta_1\beta_2$ or $(\beta\gamma)_1(\beta\gamma)_2$. For these cases, one would observe relaxation phenomena characteristic of two coupled bindings, and the curves of $1/\tau_{\beta 1}$ *vs.* S_t which would be observed would be much like those of Figure 1A, *i.e.*, they would increase without limit as S_t increases.

Case 8. The Use of the S-Buffered and Other Assumptions in a Specific Case. In their work which paved the way for the application of relaxation spectrometry to allosteric enzymes, Eigen and de Maeyer (1963) discussed some of the assumptions needed to solve complex relaxation spectra. Kirschner *et al.* (1966) found that reasonable agreement with experiment was obtained when they assumed that yeast glyceraldehyde-3-phosphate dehydrogenase followed a modified Monod–Wyman–Changeux (1965) concerted model for nonexclusive binding of a ligand to an enzyme which could exist in two conformational states. Their work incorporated the following assumptions in addition to those normally found in this model when used in equilibrium experiments. (a) All binding steps reequilibrate rapidly compared to conformational steps. This assumption has also been used here and allows the separation of relaxation rates in the analysis. (b) The intrinsic rates of association and dissociation to and from a subunit in a given conformational state are the same for a given conformation regardless of the state of occupancy of adjacent subunits. The original concerted model specifies nothing about binding or dissociation rate constants, but rather specifies equality in the appropriate intrinsic equilibrium constants. Equality of the rate constants is not a necessary feature of the Monod equilibrium model, but in fact represents a further reasonable restriction which greatly simplifies the complex mathematical manipulations. (c) The existence of binding equilibria for all states of protein in the Monod nonexclusive binding scheme implies, of course, by the cyclical nature of the free-energy function, that conformational equilibria between all states also exist. (d) In the conformational isomerizations between correspondingly liganded states, all rate constants in one direction are equal, and the difference in isomerization rates as a function of the liganded state of the enzyme is reflected in the variation of the rate constants in the opposite direction. This *ad hoc* assumption, even though it may be reasonable, is without rigorous experimental basis. This artificial constraint is not necessary, of course, and a general expression wherein it is not used has been derived by Eigen (1968). (e) In considering the conformational isomerization processes, when the equilibrium is perturbed, the per-

TABLE III: Equations for Concentration Dependence of Relaxation Rates for Cases 4–7

Concentration Dependence of Relaxation Rates for Case 4: ($\beta_1\ \gamma_1\ \beta_2\ \gamma_2$)

A. ($\beta_1''\ \gamma_1\ \beta_2''\ \gamma_2'$)

(1) Consideration of γ_1:

$$1/\tau_{\gamma 1} = \frac{2k_{\gamma 1}\left\{\left[K'_{\gamma 2}+1\right]\left[(\bar{E})\left(\frac{K_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}}+\frac{\bar{S}}{K''_{1\beta}}\right)+\frac{\bar{S}^2}{2K''_{1\beta}}\right]+\bar{E}+\bar{S}\right\}+2k_{1\gamma}\left\{\left[K'_{\gamma 2}+1\right]\left[\bar{E}\right]\left[\frac{K_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}}+\frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}}\right]+\bar{E}+\bar{S}+K_{1\beta}\right\}}{\left[K'_{\gamma 2}+1\right]\left[(\bar{E})\left(\frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{\bar{S}}{K''_{1\beta}}+\frac{K_{\gamma 1}\bar{S}^2}{K''^2_{1\beta}}\right)+\bar{S}+\frac{\bar{S}^2}{K''_{1\beta}}\right]+2(\bar{E}+\bar{S})}$$

(2) Consideration of γ_2':

$$1/\tau'_{\gamma 2} = \frac{k'_{\gamma 2}\left[(\bar{E})\left(\frac{\bar{S}}{K''_{1\beta}}+\frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{K_{\gamma 1}\bar{S}^2}{K''^2_{1\beta}}\right)+\bar{S}+\frac{\bar{S}^2}{2K''_{1\beta}}\right]+k'_{2\gamma}\left[(\bar{E})\left(\frac{\bar{S}}{K''_{1\beta}}+\frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{K_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}}+2\right)+3\bar{S}+\frac{\bar{S}^2}{2K''_{1\beta}}\right]}{(\bar{E})\left(\frac{\bar{S}}{K''_{1\beta}}+\frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{K_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}}+2\right)+3\bar{S}+\frac{\bar{S}^2}{2K''_{1\beta}}}$$

B. ($\beta_1''\gamma_1'\ \beta_2''\ \gamma_2$)

(1) Consideration of γ_2:

$$1/\tau_{\gamma 2} = \frac{k_{\gamma 2}\left\{\left[\bar{E}\right]\left[4K'_{\gamma 1}\bar{S}+\left(K'_{\gamma 1}+1\right)\frac{K'_{\gamma 1}\bar{S}^2}{K''_{1\beta}}\right]+K_{\gamma 1}\bar{S}^2\right\}}{\left[\bar{E}\right]\left[\left(K'_{\gamma 1}+1\right)\left(2K''_{1\beta}+\frac{K'_{\gamma 1}\bar{S}^2}{K''_{1\beta}}\right)+4K'_{\gamma 1}\bar{S}\right]+2K''^2_{1\beta}+2K''_{1\beta}\left(K'_{\gamma 1}+1\right)\bar{S}+K'_{\gamma 1}\bar{S}^2}+k_{2\gamma}$$

(2) Consideration of γ_1':

$$1/\tau'_{\gamma 1} = \frac{2k'_{\gamma 1}\left[(\bar{E})\left(2\bar{S}+\frac{K'_{\gamma 1}\bar{S}}{K''_{1\beta}}+2K''_{1\beta}\bar{S}+\bar{S}^2\right)\right]+4k'_{1\gamma}K''_{1\beta}\left[(\bar{E})\left(1+\frac{K'_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{K'_{\gamma 1}\bar{S}^2}{2K''^2_{1\beta}}\right)+\bar{S}+K''_{1\beta}\right]}{(\bar{E})\left(2K'_{\gamma 1}\bar{S}+\frac{\bar{S}}{K''_{1\beta}}+\frac{K'_{\gamma 1}\bar{S}^2}{K''^2_{1\beta}}+4K''_{1\beta}\right)+6K''_{1\beta}\bar{S}+2\bar{S}^2+4K''^2_{1\beta}\bar{S}}$$

Concentration Dependence of Relaxation Rates for Case 5: ($\gamma_1\ \beta_1\ \gamma_2\ \beta_2$)

A. ($\gamma_1\ \beta_1''\ \gamma_2'\ \beta_2''$)

(1) Consideration of γ_1.

$$1/\tau_{\gamma 1} = \frac{k_{1\gamma}\left\{\left[\bar{E}\right]\left[(K_{\gamma 1})(K'_{\gamma 2}+1)+\frac{2K_{\gamma 1}K'_{\gamma 2}\bar{S}}{K''_{1\beta}}\right]+K''_{1\beta}\right\}}{\left[K'_{\gamma 2}+1\right]\left[(\bar{E})\left(K_{\gamma 1}+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{2K''^2_{1\beta}}\right)+\bar{S}\right]+\frac{2K_{\gamma 1}K'_{\gamma 2}(\bar{E})(\bar{S})}{K''_{1\beta}}+\frac{K'_{\gamma 2}\bar{S}^2}{2K''_{1\beta}}+K''_{1\beta}}+k_{\gamma 1}$$

(2) Consideration of γ_2':

$$1/\tau'_{\gamma 2} = \frac{k'_{\gamma 2}\left\{\left[\bar{E}\right]\left[K_{\gamma 1}+\frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{2K''^2_{1\beta}}\right]+\frac{\bar{S}}{K''_{1\beta}}\right\}+k'_{2\gamma}\left\{\left[\bar{E}\right]\left[\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{2K''^2_{1\beta}}+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}}{K''_{1\beta}}+K_{\gamma 1}\right]+\bar{S}+K''_{1\beta}\right\}}{(\bar{E})\left(\frac{K_{\gamma 1}\bar{S}}{2K''_{1\beta}}+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}}{2K''_{1\beta}}+K_{\gamma 1}+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{2K''_{1\beta}}\right)+\frac{3\bar{S}}{2}+K''_{1\beta}+\frac{\bar{S}^2}{2K''_{1\beta}}}$$

B. ($\gamma_1'\beta_1''\ \gamma_2\ \beta_2''$)

(1) Consideration of γ_2:

$$1/\tau_{\gamma 2} = \frac{k_{\gamma 2}\left\{\left[\bar{E}\right]\left[(K'_{\gamma 1}+1)(K'_{\gamma 1})+\frac{K'^2_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{K'^2_{\gamma 1}K_{\gamma 2}\bar{S}^2}{K''^2_{1\beta}}+\frac{K_{\gamma 1}\bar{S}}{K''_{1\beta}}\right]+K'_{\gamma 1}\bar{S}+\frac{K'_{\gamma 1}\bar{S}^2}{2K''_{1\beta}}\right\}+k_{2\gamma}\left\{\left[\bar{E}\right]\left[K'_{\gamma 1}+1\right]\left[\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}^2}{K''_{1\beta}}+K'_{\gamma 1}\right]+(K'_{\gamma 1}+1)K''_{1\beta}+K'_{\gamma 1}\bar{S}\right\}}{(\bar{E})(K'_{\gamma 1}+1)\left(\frac{K'_{\gamma 1}\bar{S}}{2K''_{1\beta}}+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}}{2K''_{1\beta}}+K'_{\gamma 1}\right)+(K'_{\gamma 1}+1)\left(\frac{\bar{S}}{2}+K''_{1\beta}\right)+K'_{\gamma 1}\bar{S}+\frac{K'_{\gamma 1}\bar{S}^2}{2K''_{1\beta}}}$$

(2) Consideration of γ_1'.

$$1/\tau'_{\gamma 1} = \frac{k'_{1\gamma}\left\{(\bar{E})\left(2K'_{\gamma 1}+\frac{K'_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}}{K''_{1\beta}}\right)+\bar{S}+2K''_{1\beta}\right\}}{(\bar{E})\left(2K'_{\gamma 1}+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}}{K''_{1\beta}}+\frac{K'_{\gamma 1}\bar{S}}{K''_{1\beta}}+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}^2}{K''^2_{1\beta}}\right)+3\bar{S}+2K''_{1\beta}+\frac{\bar{S}^2}{K''_{1\beta}}}+k'_{\gamma 1}$$

TABLE III (*Continued*)

Concentration Dependence of Relaxation Rates for Case 6: ($\gamma_1 \beta_1 \beta_2 \gamma_2$)

A ($\gamma_1 \beta_1'' \beta_2'' \gamma_2'$)

(1) Consideration of γ_1

$$1/\tau_{\gamma 1} = \frac{k_{1\gamma}\left\{K''_{1\beta}K''_{2\beta} + [\bar{E}]\left[4(K'_{\gamma 2}+1)K_{\gamma 1}\bar{S} + K''_{2\beta}K_{\gamma 1}\right]\right\}}{[\bar{E}]\left[K''_{2\beta}K''_{1\beta} + (K'_{\gamma 2}+1)(4K_{\gamma 1}\bar{S}) + \frac{K_{\gamma 1}\bar{S}^2}{K''_{1\beta}}\right] + K''_{1\beta}K''_{2\beta} + K''_{2\beta}\bar{S} + (K'_{\gamma 2}+1)(\bar{S})^2} + k_{\gamma 1}$$

(2) Consideration of γ_2'

$$1/\tau_{\gamma 2}' = \frac{k'_{\gamma 2}\left\{[\bar{E}]\left[\frac{K_{\gamma 1}\bar{S}^2}{K''_{1\beta}} + 4K_{\gamma 1}\bar{S}\right] + \bar{S}^2\right\}}{(\bar{E})\left(4K_{\gamma 1}\bar{S} + \frac{K_{\gamma 1}\bar{S}^2}{K''_{1\beta}} + K''_{2\beta}K_{\gamma 1}\right) + K''_{1\beta}K''_{2\beta} + K''_{2\beta}\bar{S} + \bar{S}^2} + k'_{2\gamma}$$

B ($\gamma_1' \beta_1'' \beta_2'' \gamma_2$)

(1) Consideration of γ_2:

$$1/\tau_{\gamma 2} = \frac{k_{\gamma 2}\left\{[\bar{E}]\left[4(K'_{\gamma 1}+1)K'_{\gamma 1}\bar{S} + \frac{K'_{\gamma 1}\bar{S}^2}{K''_{1\beta}}\right] + \bar{S}^2\right\}}{[\bar{E}]\left[(K'_{\gamma 1}+1)(4K'_{\gamma 1}\bar{S} + K''_{2\beta}K'_{\gamma 1}) + \frac{K'_{\gamma 1}\bar{S}^2}{K''_{1\beta}}\right] + (K'_{\gamma 1}+1)(K''_{1\beta}K''_{2\beta}) + K''_{2\beta}\bar{S} + \bar{S}^2} + k_{2\gamma}$$

(2) Consideration of γ_1':

$$1/\tau_{\gamma 1}' = \frac{k'_{1\gamma}\left\{[\bar{E}][4K'_{\gamma 1}\bar{S} + K''_{2\beta}K'_{\gamma 1}] + K''_{1\beta}K''_{2\beta}\right\}}{[\bar{E}]\left[4K'_{\gamma 1}\bar{S} + \frac{K'_{\gamma 1}\bar{S}^2}{K''_{1\beta}} + K''_{2\beta}K'_{\gamma 1}\right] + K''_{1\beta}K''_{2\beta} + K''_{2\beta}\bar{S} + \bar{S}^2} + k'_{\gamma 1}$$

Concentration Dependence of Relaxation Rates for Case 7: ($\beta_1 \gamma_1 \gamma_2 \beta_2$)

A ($\beta_1'' \gamma_1 \gamma_2' \beta_2''$)

(1) Consideration of γ_1.

$$1/\tau_{\gamma 1} = \frac{k_{\gamma 1}\left\{[\bar{E}]\left[K''_{2\beta}(1+K'_{\gamma 2})\left(1+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{K''_{1\beta}K''_{2\beta}}\right)+K'_{\gamma 2}\bar{S}\right]+K''_{2\beta}(1+K'_{\gamma 2})\bar{S}+K'_{\gamma 2}\bar{S}^2\right\}+k_{1\gamma}\left\{[\bar{E}]\left[K'_{2\beta}\left(1+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{K''_{1\beta}K''_{2\beta}}\right)+K_{\gamma 1}K'_{\gamma 2}\bar{S}\right]+K''_{1\beta}K''_{2\beta}+K''_{2\beta}\bar{S}\right\}}{[\bar{E}]\left[(1+K'_{\gamma 2})\left(1+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{K''_{1\beta}K''_{2\beta}}+K_{\gamma 1}K'_{\gamma 2}\bar{S}\right)+K'_{\gamma 2}\bar{S}\right]+[K''_{2\beta}(1+K'_{\gamma 2})+K'_{\gamma 2}]\bar{S}+(1+K'_{\gamma 2})K''_{1\beta}K''_{2\beta}+K'_{\gamma 2}\bar{S}^2}$$

(2) Consideration of γ_2'

$$1/\tau_{\gamma 2}' = \frac{k'_{\gamma 2}\left\{[\bar{E}]\left[K''_{2\beta}+(1+K_{\gamma 1}K'_{\gamma 2})\bar{S}+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{K''_{1\beta}}\right]+(K''_{1\beta}+K''_{2\beta})\bar{S}+\bar{S}^2+K''_{1\beta}K''_{2\beta}\right\}+k'_{2\gamma}\left\{[\bar{E}]\left[K''_{2\beta}+K_{\gamma 1}K'_{\gamma 2}\bar{S}+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{K''_{1\beta}}\right]+K''_{1\beta}K''_{2\beta}+K''_{2\beta}\bar{S}\right\}}{[\bar{E}]\left[(1+K_{\gamma 1}K'_{\gamma 2})\bar{S}+\frac{K_{\gamma 1}K'_{\gamma 2}\bar{S}^2}{K''_{1\beta}}+K''_{2\beta}\right]+\bar{S}^2+K''_{1\beta}K''_{2\beta}+(K''_{1\beta}+K''_{2\beta})\bar{S}}$$

B. ($\beta_1'' \gamma_1' \gamma_2 \beta_2''$)

(1) Consideration of γ_2.

$$1/\tau_{\gamma 2} = \frac{k_{\gamma 2}K'_{\gamma 1}\left\{[\bar{E}]\left[K''_{2\beta}+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}^2}{K''_{1\beta}}+\bar{S}\right]+K''_{2\beta}\bar{S}+\bar{S}^2\right\}+k_{2\gamma}\left\{[\bar{E}]\left[K''_{2\beta}(1+K'_{\gamma 1})\left(1+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}^2}{K''_{1\beta}K''_{2\beta}}\right)+K_{\gamma 2}K'_{\gamma 1}\bar{S}\right]+K''_{2\beta}(K'_{\gamma 1}+1)\bar{S}+K''_{1\beta}K''_{2\beta}\right.}{[\bar{E}]\left[(K'_{\gamma 1}+1)(K''_{2\beta})\left(1+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}^2}{K''_{1\beta}K''_{2\beta}}\right)+(1+K'_{\gamma 1})\bar{S}+K_{\gamma 1}K_{\gamma 2}\bar{S}\right]+[K''_{2\beta}(1+K'_{\gamma 1})+K''_{1\beta}]\bar{S}+K''_{1\beta}K''_{2\beta}+(1+K'_{\gamma 1})\bar{S}^2}$$

(2) Consideration of γ_1'

$$1/\tau_{\gamma 1}' = \frac{k'_{\gamma 1}\left\{[\bar{E}]\left[K''_{2\beta}+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}^2}{K''_{1\beta}}+\bar{S}\right]+K''_{2\beta}\bar{S}+\bar{S}^2\right\}}{[\bar{E}]\left[K''_{2\beta}+\frac{K'_{\gamma 1}K_{\gamma 2}\bar{S}^2}{K''_{1\beta}}+(K'_{\gamma 1}K_{\gamma 2}+1)\bar{S}\right]+[K''_{2\beta}+K''_{1\beta}]\bar{S}+\bar{S}^2+K''_{1\beta}K''_{2\beta}}+k_{1\gamma}$$

turbation in $\bar{S}$ is assumed to be so small compared with that of the other concentration variables of the system that it may be set to zero (the "S-buffered" approximation). The approximation is valid either when $S_t \ll E_t$ or when $S_t \gg E_t$. Over a very important practical working range to concentration, namely, that in which $S_t \sim E_t$, one can show that the "S-buffered" approximation does not hold, although the types of curves one might predict in the absence of the assumption can often resemble those predicted with the assumption (Eigen, 1968). This approximation offers the advantages of (1) expressing the relaxation rate of conformational processes as a function of $\bar{S}$ only (the concentration of enzyme forms does not appear in the rate expression), (2) simplifying the algebra from a totally unwieldy form in the absence of the approximation to a very compact form, and (3) allowing one to write the general expressions for an n-subunit enzyme.

TABLE IV Comparison of the Concentration Dependence of the Conformational Relaxation Rates for Various Mechanisms under the "S-Buffered" Assumption

A. Mechanism $\beta_1'' \gamma_1' \beta_2'' \gamma_2$ (Table I)

$$1/\tau_{\gamma 2} = \frac{(k_{\gamma 2}K'_{\gamma 1} + k_{2\gamma}K'_{\gamma 1})\bar{S}^2 + 2(k_{2\gamma}K''_{1\beta} + k_{2\gamma}K'_{\gamma 1}K''_{1\beta})\bar{S} + 2k_{2\gamma}K''^2_{1\beta}}{K'_{\gamma 1}\bar{S}^2 + K''_{1\beta}(K'_{\gamma 1} + 1)\bar{S} + 2K''^2_{1\beta}}$$

B Mechanism $\gamma_1'\beta_1''\gamma_2\beta_2''$ (Table III)

$$1/\tau_{\gamma 2} = \frac{K'_{\gamma 1}[k_{\gamma 2} + k_{2\gamma}]\bar{S}^2 + K''_{1\beta}[2K'_{\gamma 1}k_{\gamma 2} + K''_{1\beta}(1 + K'_{\gamma 1})k_{2\gamma}]\bar{S}}{K'_{\gamma 1}\bar{S}^2 + [K''_{1\beta}(1 + K'_{\gamma 1}) + 2K''_{1\beta}K'_{\gamma 1}]\bar{S} + 2K''^2_{1\beta}(1 + K'_{\gamma 1})}$$

C Mechanism $\gamma_1' \beta_1'' \beta_2'' \gamma_2$ (Table III)

$$1/\tau_{\gamma 2} = \frac{K'_{\gamma 1}(k_{\gamma 2} + k_{2\gamma})\bar{S}^2 + k_{2\gamma}K''_{1\beta}K'_{\gamma 1}\bar{S} + k_{2\gamma}K''_{1\beta}K''_{2\beta}(1 + K'_{\gamma 1})}{K'_{\gamma 1}\bar{S}^2 + K''_{1\beta}K'_{\gamma 1}\bar{S} + K''_{1\beta}K''_{2\beta}(1 + K'_{\gamma 1})}$$

D. Modified Monod Mechanism (notation and assumptions are those of Kirschner, et al.(2)) applied to a two subunit case.

$$1/\tau_3 = \frac{(k'_0 + k_0c^2)\bar{S}^2 + 2(k'_0 + k_0c)\bar{S}K + (k'_0 + k_0)K^2}{\bar{S}^2 + 2\bar{S}K + K^2}$$

All relaxations are of the form:

$$1/\tau = \frac{A\bar{S}^2 + B\bar{S} + C}{D\bar{S}^2 + E\bar{S} + F}$$

These assumptions, as necessary as they may be for managing the derivation of the rate expressions, must be considered carefully when a mechanism is said to be "proved" by relaxation spectrometry. For example, in Table IV, we have applied the "S-buffered" assumption to some cases considered in Tables I and II, as well as adapting the expressions previously derived by Kirschner *et al.* (1966) and Eigen (1968) for the concerted model applied to a two-subunit enzyme.

Results

Limitations of Diagnostic Approach. One of the most impressive conclusions to be derived from Table II is that the same type of relaxation spectra is obtained from many mechanisms. For example, a reciprocal relaxation time which decreases with increasing S_t approaching a plateau value (curve g of Figure 1) is obtained by a monomer with a prior dissociation step (case 3), and several types of dimer sequential models (cases 4, 5, 6, and 7). This result is typical rather than atypical and hence a particular type of relaxation curve will rarely be diagnostic for a mechanism. This conclusion is reinforced by the facts that (a) these curves are only obtained after severely limiting assumptions, (b) experimental errors in the relaxation data (usually 10–25%) would make many curves which are distinguishable theoretically indistinguishable experimentally, and (c) the variety of curves shown in Figure 1 and Table II come from a dimer, which will have far fewer adjustable parameters than tetramers and larger proteins.

This conclusion is further reinforced by the comparison of the expected results from the concerted and sequential models shown in Table IV. We see from this table that all the mechanisms considered there lead to the same relaxation expression and are kinetically indistinguishable. The concerted mechanism, of course, predicts some limitation on the relationships between various constants but this could not exclude a more general mechanism. Janin and Iwatsubo (1969) recently studied the threonine-sensitive aspartokinase I–homoserine dehydrogenase and found the relaxation processes which were consistent with a concerted process. As we can see from Table IV, the sequential model is also compatible with the relaxations they observed and therefore no distinction between models can be made based on their data.

Another diagnostic procedure which has been used to distinguish mechanisms is to consider the number of relaxation processes allowed. The Monod model leads logically to the assumption that a number of binding processes will occur with the same velocity. Similarly, in the simplest sequential models (Koshland *et al.*, 1966), binding in more than one step may logically proceed at rates which are the same within experimental error when the processes are highly analogous, *i.e.*, the change in relation to adjacent subunits is similar. Thus, one may be tempted to discard mechanisms at times on the basis of finding only three relaxation effects when an alternative pathway predicts four.

The situation is not so simple in practice. Often, relaxations may be present in a system which occur at rates too great to be observed with the instrument at hand. Sometimes these processes are coupled to slower processes, and one can infer their presence from the concentration dependence of slower processes, but experimental limitations are such that one cannot be sure that he can always infer the presence of all processes. As an example, it would be very difficult distinguishing two rapid binding steps from four rapid binding steps if one had to infer their existence merely from the concentration dependence of a conformational relaxation. As illustrated above, rapid conformational steps can not *ipso facto* be inferred from slower binding relaxations (*cf.* eq 4). Thus, the relaxation spectrum of a two-subunit enzyme which follows a sequential model could appear deceptively similar to that of an enzyme following a concerted pattern if one of the conformational processes was much faster than the binding and the other was much slower.

There also exists the possibility that relaxations are present which are not kinetically significant to the binding mechanism

under study. Suppose, for example, that an enzyme isomerizes between two or more states in either a sequential or a concerted manner, but that the multiple states have identical binding properties. One should observe in this case a relaxation process which is concentration invariant, and thus difficult to interpret. Conversely, if one sees an apparently concentration invariant process near the rapid time resolution limit of the instrument, one cannot be sure that this curve could not also be the high-S_t plateau of the relaxation rate that would increase at lower S_t to a value beyond the fast relaxation limit of the instrument. It goes without saying that one may observe $1/\tau$ *vs.* S_t curves which change very little over the entire range of S_t simply because the low S_t and high S_t limits of the curves do not differ much. Within a typical 10–20% error, these curves would appear not to vary at all with S_t, and one might consider these processes to be mechanistically irrelevant when, in fact, they are quite significant.

Certain features of experimental $1/\tau$ *vs.* S_t curves may be obscured by weak amplitudes. It is entirely possible that many relaxation processes may exist in an enzyme in the accessible time range which do not lead to a detectable relaxation effect. One must therefore regard the number of relaxation phenomena observed as the minimum number which exist in the system.

Other authors, notably Czerlinski (1967) and Schuster and Ilgenfritz (1969), have pointed out some of the limitations of relaxation techniques particularly when the full concentration range is not explored.

In view of the above derivations it seems very improbable that temperature-jump data alone can exclude any model in proteins containing four or more subunits.

It should be emphasized that the simplest sequential model has the same number of independent parameters as the exclusive concerted model. Once qualifying assumptions are made to make one model fit the data, it is necessary to attempt equivalently qualifying assumptions for the alternative in order to test its ability to cope with the data. Unless this is done, no conclusion of sequential *vs.* concerted mechanisms can be made, and even when it is done, the conclusions only apply if the series of assumptions ultimately prove to be valid.

Assets of Relaxation Techniques. Although it is clear that a variety of analytical expressions can be obtained from simple changes in a dimer, some general features emerge from the survey in Table II. For these specific models, the following hold true. (a) All relaxation rates due to binding processes increase without limit as S_t becomes large, although some may go through local minima. (b) If a conformational process occurs at the beginning of a sequence (as in Tables II and III), it will always be characterized by a relaxation rate which decreases with increasing S_t. (c) If a conformational process terminates a sequence (as in Tables I and III), it will be characterized by a relaxation rate which increases with increasing S_t. One will notice, in the development of eq 7 and 10 for a one-subunit enzyme, that the conformational process also terminates the reaction sequence. This equation, however, predicts the possibility of an initial decrease of $1/\tau$ *vs.* S_t, despite a net increase in $1/\tau$ between the limits of low and high S_t, a fact which implies that this curve should show a local minimum. This one case would appear to violate generalization (c). One can show that this decrease will occur only when $E_t \gg K_{1\beta}'$, and that it occurs in a region of substrate concentration which would lead to very low amplitudes in the relaxation curve. This assumes that extinction coefficient change and/or ΔH° for the step in question is not abnormally high. When $E_t \leq K_{1\beta}'$, the initial slope is zero, and a minimum in the curve is not predicted. Thus, the case of a one-subunit enzyme also will be observed in most cases to follow this generalization over the range of substrate concentration within which relaxation effects are observable. This generalization appears to hold, therefore, for any number of subunits. It is important to note that such an analysis might also suggest that maxima or minima in the low S_t portion of other $1/\tau$ *vs.* S_t curves may also be obscured by low amplitudes. (d) If a conformational process occurs in the middle of a sequence (as in Tables II and IV), whether a net increase or decrease in the relaxation rate is observed depends on the relative magnitudes of the various constants of the system with respect to each other and with respect to E_t. Maxima and minima may also be observed in these cases, if amplitudes are sufficiently strong in the appropriate regions. (e) Relaxation rates due to unimolecular isomerization processes always asymptotically approach a limiting value at high S_t.

These generalizations apply, of course, where the assumptions used in the derivations of the equations are valid, namely, when the binding processes are rapid relative to isomerizations events and when the relaxation times are distinct. These are very restrictive assumptions and therefore the curves obtained must be viewed only as clues to the design of future experiments, not as proofs of mechanism. Such preliminary hypotheses are useful, however, because the rigorous solution of all the potential equations is a Herculean task, impossibly time consuming for ordinary studies. Furthermore, quantitative relationships between constants may reinforce hypotheses based on the shapes of the curves.

Application to Other Systems. It is likely that more subtle generalizations will become obvious upon closer examination of the relaxation rate curves. Using the conclusions for the dimer, however, we can suggest the general appearance of the relaxation spectra characteristic of the conformational events for a four-subunit enzyme whose binding mechanism would be abbreviated $\beta_1\gamma_1\beta_2\gamma_2\beta_3\gamma_3\beta_4\gamma_4$, when the binding ($\beta$) steps relax more rapidly than the conformational (γ) steps. The terminal step will be characterized by an increasing $1/\tau$ *vs.* S_t curve, with an asymptotic limit, and the other three conformational steps will approach some limiting value of $1/\tau$ *vs.* S_t at high S_t, although the precise nature of the concentration dependence depends on relative rates of the various processes and the magnitudes of the constants involved. For the sequence $\gamma_1\beta_1\gamma_2\beta_2\gamma_3\beta_3\gamma_4\beta_4$, the initial conformational process will decrease to a limiting value at high S_t, and the other conformational processes will have concentration dependencies which we can only say, without further information, will approach asymptotic limiting values. All binding processes will show relaxation effects which increase without limit in rate as S_t becomes large. Thus, the qualitative reasoning used in the cases of the dimeric protein allow us some clue to the type of results which will occur for proteins with more than two interacting sites.

It is worth noting that the processes which we have called bindings, and symbolized by β, and the processes called conformational changes, and symbolized by γ, are actually bimolecular (enzyme form + S) and unimolecular processes, respectively. Nothing in the mathematics, of course, requires that these processes be precisely what we have postulated here unless we have independent evidence that such an event is transpiring. This has both advantages and disadvantages. The disadvantage is that a process can give relaxation rate data which agree precisely with a given model, but the molecular events could be different from those postulated. For example, the mathematics of a $\beta_1\gamma_1\beta_2\gamma_2$ model could also be obtained

if binding steps at nonproductive sites were followed by a position isomerization of substrate to a productive site. A similar pattern could be obtained if isomerization of substrate on the surface of the enzyme followed binding. The γ_1 and γ_2 steps in these cases would still be unimolecular isomerization steps, and the mathematics does not distinguish between the various alternatives. These ambiguities also have an advantage since the mathematics derived here will then hold for all processes in which the β steps are bimolecular and the γ steps are unimolecular, irrespective of the detailed molecular events which are involved in these processes. Independent methods, *e.g.*, using dye molecules, protein probes, nmr, or reporter groups, may in some cases make it possible to obtain independent evidence in regard to the nature of the individual events. However, the cautions mentioned in the previous section apply even more forcefully as the number of interacting sites in the protein increases.

It has been known for some time (Eigen and de Maeyer, 1963) that the concentration dependence of amplitudes, or intensities of relaxation rate traces, may also be used in mechanistic diagnosis. Instruments must be very carefully calibrated, and they must be very stable for such work, and meaningful quantitative results are obtained only after very tedious experimentation. For these reasons, we have dwelt primarily on the use of the concentration dependence of $1/\tau$ as a diagnostic tool. With increasing instrumental sensitivity, however, the interpretation of experimental intensities will probably become more useful. Clever applications of this parameter in fact have recently been reported (Guillain and Thusius, 1970; Winkler, 1969).

Conclusion

Relaxation spectrometry is a powerful tool for elucidating the behavior of allosteric proteins. Other diagnostic techniques can be used for equilibrium processes, but the understanding of very fast rate processes requires the use of this experimental tool. The problem with relaxation spectrometry for allosteric proteins is that the mathematics for even the simplest cases becomes very complex. As shown above, it is not possible to write a rigorous mathematical general formulation for the simplest allosteric system, *i.e.*, a dimer, involving the interaction of two subunits. If one makes assumptions which simplify the alternatives, *e.g.*, only two conformations per subunit, relaxation times are separable, etc., one can obtain analytical solutions and determine the shapes of the relaxation spectra. Even in those cases, however, it is found that distinction between a concerted and a sequential model is difficult. In many of the cases the same diagnostic curves are obtained for both models. In other cases, one cannot be sure that the experimental observations are complete, *e.g.*, relaxation times may be obscured by degeneracy or missed by being outside the range of detection. This does not mean that the two models are indistinguishable under all circumstances but it does suggest that it will be almost impossible from the relaxation spectrometry alone to prove a model even for a dimer. It follows that proteins containing a greater number of subunits, *i.e.*, four or six, will allow enough variables to prevent distinction between a concerted and sequential mechanism by fast reaction techniques.

A limitation in the diagnostic value of a technique is not unique to relaxation spectrometry. In fact, other diagnostic tests such as Hill plots, equilibrium dialysis, etc., also have limitations and can rarely be used alone to establish an individual model (Koshland, 1970). Because of this complexity of the mathematics, however, the temptation exists to find one mechanism compatible with the relaxation data and imply that alternatives are excluded. The above derivations emphasize the importance of making systematic permutations for models of similar degrees of complexity before one alternative is selected as preferable.

The above limitation of relaxation spectrometry, however, does not mean that it is not an exceptionally powerful tool. It is the only way of measuring processes too fast to observe in the stopped-flow machine and it can give extraordinary insight into the interactions between protein subunits. Two obvious ways of reducing the number of alternative mechanisms is to identify through spectral or other means some rate processes and then determine their magnitude by relaxation spectrometry. Reporter groups may be particularly effective in this regard. Secondly, the diagnostic rule-of-thumb procedures, such as those used illustratively above for a dimer, can be used as a first working hypothesis to design and select the next tools to establish the postulated mechanism. It must be recognized that these diagnostic procedures will be an indication of the processes occurring only if all of the assumptions in the derivations of the equations hold. The assumptions are not necessarily correct but they may be. They can certainly serve as a potential basis for further experiments. Thus, relaxation spectra of the type illustrated in Figure 1 or Table II can be used with binding curves and reactivity data to narrow available alternatives. Equilibrium dialysis curves combined with relaxation spectra, for example, should limit alternatives since the equilibrium methods will demand certain forward and reverse rate ratios.

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Role of an Allosteric Effector. Guanosine Triphosphate Activation in Cytosine Triphosphate Synthetase*

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ABSTRACT: The allosteric effector, GTP, acts to accelerate the synthesis of CTP by the enzyme, CTP synthetase, when glutamine is the nitrogen source.

$$\text{Glu-NH}_2 + \text{UTP} + \text{ATP} \xrightarrow{\text{GTP}} \text{Glu} + \text{CTP} + \text{ADP} + \text{P}_i$$

GTP accelerates the glutaminase activity of the enzyme but has little or no effect when NH_3 is the nitrogen source. Kinetic studies indicate that the role of GTP can be explained by its effect in accelerating the formation of glutamyl-enzyme from the E·Gln Michaelis complex. ATP and UTP, the substrates of the NH_3 to CTP reaction, also act as allosteric effectors for the glutaminase step in the enzyme mechanism. GTP binding was found to be noncooperative in the dimer, negatively cooperative in the tetramer at high temperatures, and positively cooperative in the tetramer at low temperatures.

Allosteric effectors are known to exert their control on an enzyme without being consumed themselves. It is now widely accepted that such effectors operate through conformational changes although the precise mechanism of these changes is not known. The similarity of these changes to a large number of other biological processes such as the peeling of a repressor from DNA, the activation of a nerve receptor molecule, and the induction of conformation changes in transport proteins suggests a widespread occurrence of properties analogous to those of the allosteric effector.

It is of particular interest, therefore, to examine the mode of reaction of an allosteric effector in a system in which the individual components have been analyzed extensively. In such a system it may be possible to dissect the overall activation or inhibition and to determine the precise mechanism by which the effector exerts its effect on the reaction pathway. CTP synthetase offered an unusual opportunity for such an examination since both the covalent chemistry of the individual steps in the reaction catalyzed by the enzyme (Levitzki and Koshland, 1971a) and the structure of the enzyme have been studied extensively (Long *et al.*, 1970). In this paper, we shall describe the role of the allosteric effector, GTP, in terms of the known chemistry and structure of the enzyme.

Experimental Section

All compounds used were of the highest purity commercially available. The ATP analog with a nitrogen atom in the β,γ position (ADPNP) was kindly donated by Dr. Ralph Yount of Washington State University, Pullman, Wash. [^{3}H]-GTP and [^{14}C]GTP in 50% ethanol were obtained from New England Nuclear Corp. Over 94% of the radioactivity was identified as GTP using thin-layer chromatography (tlc) on PEI-cellulose[1] (Randerath, 1964).

CTP synthetase was purified from *Escherichia coli* B using a modification (Levitzki and Koshland, 1970) of the method described earlier (Long and Pardee, 1967; Long *et al.*, 1970). Protein was determined by the method of Lowry *et al.* (1951). Inorganic phosphate was determined according to Fiske and Subbarow (Leloir and Carolina, 1957). Nucleotide concentrations were determined spectrophotometrically (National Academy of Sciences, 1960). γ-Glutamyl hydroxamate was determined using the $FeCl_3$–HCl reaction (Pamiljans *et al.*, 1962) and ammonia was determined using the glutamic dehydrogenase submicro method (Levitzki, 1970).

Binding Measurements by Equilibrium Dialysis. Equilibrium dialysis was carried out in dialysis cells of the design of Englund *et al.* (1970) having a volume of 30 μl in each compartment. Their aliquoting method (Englund *et al.*, 1970) was used except that aliquots of 3 μl were taken instead of 4 μl. This was found to increase the reproducibility of the sampling so that an accuracy of better than 1% was obtained. The membranes used were size 20 dialysis tubing (Union Carbide) boiled three times for 5 min in 1 mM EDTA and stored in 0.1 mM EDTA at 4°.

Binding Measurements Using Paulus Filtration Method. The binding by the Paulus filtration method (Paulus, 1969) was found to be extremely useful. However, the method must be modified for some cases since [^{14}C]GTP binds to Diaflo membranes (UM-10) in the absence of protein. This binding to the membrane follows a Michaelis–Menten isotherm with an apparent dissociation constant of 1.25×10^{-4} M. The fraction of [^{14}C]GTP bound is 20% of the amount

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[1] Abbreviations used are: polyethylenimine, PEI; 6-diazo-5-oxonorleucine, DON; β,γ-NH-ATP, ADPNP; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, Hepes.