Allosteric Interactions in Aspartate Transcarbamylase. III. Interpretation of Experimental Data in Terms of the Model of Monod, Wyman, and Changeux*

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ABSTRACT: A detailed analysis according to the two-state model of Monod, Wyman, and Changeux (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88) has been made of data obtained for the allosteric interactions of aspartate transcarbamylase from *Escherichia coli*. The data presented in papers I and II of this series are quantitatively consistent with the equations of the two-state model and are fit by a single set of values for the parameters. These values suggest that (1) the substrate analog succinate is bound *exclusively* (at least 10^3-fold more strongly) by one of the putative states of the enzyme, while (2) the feedback inhibitor cytidine triphosphate is bound only *preferentially* (1.7-fold more strongly) by the other state of the enzyme. (3) In the absence of effectors, the distribution of enzyme molecules between these states favors in a 4:1 ratio the state to which the inhibitor binds preferentially and the substrate does not bind. A comparison is made between the extent of ligand binding and the extent of alterations in the protein conformation of the enzyme over a wide range of concentrations of substrate and feedback inhibitor analogs (data presented in papers I and II of this series). The conformational alterations are found to approach completion at concentrations of substrate analog which only partially saturate the specific binding sites of the enzyme (*e.g.*, 50% completion when only 15% of the sites is filled). This observation is compatible with the two-state model but not with certain alternative models.

In the first and second papers of this series (Changeux *et al.*, 1968; Gerhart and Schachman, 1968), experimental data have been presented which indicate that (1) aspartate transcarbamylase from *Escherichia coli* (ATCase;[1] carbamyl phosphate:L-aspartate carbamyl transferase, EC 2132) mediates indirect, *i.e.*, allosteric, interactions among substrate and/or inhibitor molecules which bind at topographically distinct sites on the enzyme surface, and (2) the binding of these ligands is accompanied by changes in the protein conformation. The objective of this paper is the detailed comparison of these observations with the predictions of the model for allosteric interactions proposed by Monod *et al.* (1965).

This model was designed primarily to account for a certain class of cooperative interactions observed for the binding of identical stereospecific ligands to regulatory enzymes (homotropic effects).[2] The mediation of these interactions requires strictly that the protein be composed of subunits. The model of Monod *et al.* represents an attempt to relate the observed cooperativity in ligand binding to the cooperativity of macromolecular structure. Accordingly, an allosteric protein is postulated to comprise a small number of identical subunits called protomers and to equilibrate between a small number of conformational states which differ in their affinity for ligands. Among these states, the most stable and consequently predominant states are those in which all protomers of the protein molecule have the same conformation (*i.e.*, symmetrical states).[3] Cooperativity in ligand binding then arises from a coordinated transition of all protomers of a protein molecule to the conformational state for which the ligand has greater affinity.

In contrast with the postulated all-or-none nature of the conformational change of the protein, the binding of a ligand molecule to a site on a subunit in a particular conformation is independent of the occupancy of other sites on the same or different subunits. The progressive changes in the enzyme conformation as a function

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[1] Abbreviations used: ATCase, aspartate transcarbamylase; ATP, adenosine triphosphate; BrCTP, 5-bromocytidine triphosphate; CTP, cytidine triphosphate; PMB, *p*-mercuribenzoic acid; Succ, succinate.

[2] For nomenclature, see paper I.

[3] An analogous two-state model for the cooperative conformational transitions in fibrous proteins has been described by Hill (1952).

of the free ligand concentration are consequently not expected to coincide with the progressive saturation of sites for that ligand. In other words, there should not exist a strict linear relationship between the fraction of sites occupied (the binding function, $\bar{Y}$) and the fraction of molecules in a given conformation (the conformational state function, $\bar{R}$). This prediction and the underlying assumption that the conformational equilibrium preexists the binding of effectors, distinguish the model of Monod *et al.* from other models in which the conformational changes are assumed to be induced by and thus coincident with ligand binding (Induced Fit Theory) (Adair, 1925a,b; Pauling, 1935; Koshland, 1963). The comparison of the observed binding of specific ligands to ATCase (paper I) with the corresponding conformational alterations of the protein (paper II) thus provides a critical test of which interpretation is more appropriate in the case of this enzyme and the considered effectors. The results of the present investigation are consistent with the two-state model of Monod *et al.*

Theory

Analytical Properties of the Model. The model of Monod *et al.* interprets allosteric interactions between ligand molecules bound at distinct sites of a protein in terms of transitions between two conformational states labeled R and T. The detailed predictions of this model are embodied in two analytical functions: the binding function ($\bar{Y}$) which represents the fraction of specific sites in the total protein population occupied by the considered ligand, and the state function ($\bar{R}$) which represents the fraction of protein molecules in the R conformation.

$$\bar{Y} = \frac{\alpha(1+\alpha)^{n-1} + L'\alpha c(1+\alpha c)^n}{(1+\alpha)^n + L'(1+\alpha c)^n} \quad (1)$$

$$\bar{R} = 1 - \bar{T} = \frac{(1+\alpha)^n}{(1+\alpha)^n + L'(1+\alpha c)^n} \quad (2)$$

where

$$L' = \left(\frac{\bar{T}}{\bar{R}}\right)_{\alpha=0} = L\prod_i \left(\frac{1+\beta_i d_i}{1+\beta_i}\right)^n \prod_j \left(\frac{1+\gamma_j e_j}{1+\gamma_j}\right)^n \quad (3)$$

is the *apparent* conformational equilibrium constant in the absence of substrate, and

$$L = \left(\frac{\bar{T}}{\bar{R}}\right)_{\alpha,\beta_i,\gamma_j=0}$$

is the *intrinsic* conformational equilibrium constant. The normalized concentrations (Greek letters) and ratios of microscopic dissociation constants from each of the two states for the substrate (S), inhibitor(s) (I_i), and activator(s) (A_j), are designated by the symbols

$$\alpha = S/k_{RS} \quad \beta_i = I_i/k_{RI_i} \quad \gamma_j = A_j/k_{RA_j}$$

$$c = k_{RS}/k_{TS} \quad d_i = k_{RI_i}/k_{TI_i} \quad e_j = k_{RA_j}/k_{TA_j}$$

where the various k_R and k_T are the microscopic dissociation constants of the considered ligand from the R and T states, respectively. For example, k_{RS} designates the microscopic dissociation of the substrate from the R state. It is assumed that the state labeled R has higher affinity for the substrate and activators ($c, e_j, < 1$), that state T has higher affinity for inhibitors ($d_i > 1$), and that n, the number of equivalent independent binding sites for each type of ligand, corresponds to the number of identical subunits (protomers). The derivations of eq 1–3 have been discussed in detail elsewhere (Monod *et al.*, 1965; Rubin and Changeux, 1966).

As pointed out by Rubin and Changeux (1966), the accessible range of values of the state function $\bar{R}$ is determined by the intrinsic allosteric constant (L) and by the ratios of the microscopic dissociation constants of the considered ligands for the two states (c, d_i, and e_j). In the absence of inhibitors or activators ($\beta = \gamma = 0$), the limits on $\bar{R}$ as a function of the relative substrate concentration $\alpha = S/k_{RS}$ are

$$\begin{matrix} \alpha = 0 & & \alpha \longrightarrow \infty \\ 1/(L+1) \leq & \bar{R} & \leq 1/(Lc^n + 1) \end{matrix} \quad (4)$$

while in the presence of saturating levels of inhibitor ($\beta \rightarrow \infty$, $\gamma = 0$) the accessible values of $\bar{R}$ are defined by

$$\begin{matrix} \alpha = 0 & & \alpha \longrightarrow \infty \\ 1/(Ld^n + 1) \leq & \bar{R} & \leq 1/(Lc^n d^n + 1) \end{matrix} \quad (5)$$

With few exceptions, these limits on the state function are different from zero and unity which are the limiting values of the saturation function ($\bar{Y}$).[4] To facilitate the comparison of conformational and ligand binding studies, it is therefore convenient to introduce a relative state function ($\bar{Rr}$). While eq 6 is more complicated

$$\bar{Rr} = \frac{\bar{R}(\alpha) - \bar{R}(\alpha = 0)}{\bar{R}(\alpha \longrightarrow \infty) - \bar{R}(\alpha = 0)} = \frac{(L'c^n + 1)\,[(1+\alpha)^n - (1+\alpha c)^n]}{(1-c^n)[(1+\alpha)^n + L'(1+\alpha c)^n]} \quad (6)$$

than the expression for $\bar{R}$ (eq 2), the function $\bar{Rr}$ has the advantage that it corresponds to the variation of a parameter of protein conformation between the limiting values measured in the absence of a specified ligand

[4] In the absence of inhibitors or activators ($\beta = \gamma = 0$) and for finite values of L, the lower limit of $\bar{R}$, $\bar{R}(\alpha = 0)$ is greater than zero, while the upper limit $\bar{R}(\alpha \rightarrow \infty)$ is unity for $c = 0$. When $\beta = \gamma = 0$ and $L \rightarrow \infty$, $\bar{R}(\alpha = 0)$ is zero but $\bar{R}(\alpha = \infty)$ is less than unity for $c > 0$ or unspecified for $c = 0$. Analogous conditions pertaining in the presence of saturating inhibitor ($\beta \rightarrow \infty$) are obtained by replacing L of the preceding statements by Ld^n.

and at saturating concentrations of the ligand. The relative state function ($\bar{R}r$), like the saturation function ($\bar{Y}$), varies between zero and unity.

In the case of a hypothetical monomer ($n = 1$), which may exhibit heterotropic but not homotropic allosteric effects, $\bar{R}r$ and $\bar{Y}$ become analytically identical although $\bar{R}$ and $\bar{Y}$ are still distinct. For oligomers ($n > 1$), on the other hand, the curve for the relative state function may precede the binding function, intersect it, or follow it as the ligand concentration is increased, depending on the particular values of n, L', and c. Explicitly, under conditions such that $L' = c^{-n/2}$, the functions $\bar{R}r$ and $\bar{Y}$ cross at their midpoint; for L' less than $c^{-n/2}$, $\bar{R}r$ intersects $\bar{Y}$ below the midpoint or precedes it; and for L' greater than $c^{-n/2}$, $\bar{R}r$ intersects $\bar{Y}$ above the midpoint.

Method of Analysis of Ligand Binding Data. The quantitative analysis of the results of equilibrium dialysis studies on ATCase (paper I) in terms of eq 1 is justified by the striking resemblance of these data to the theoretical saturation functions computed by Monod *et al.* (1965). For the evaluation of the various parameters, it was found convenient to represent the binding data in the coordinate system introduced by Scatchard (1949), in which the number of ligand molecules bound per molecule of protein divided by the free ligand concentration ($r/S = n\bar{Y}/S$) is plotted against $n\bar{Y} = r$. When there is significant cooperativity of ligand binding, this representation of the data gives a curve which is convex with respect to the abscissa and from which the four parameters of the two-state model (n, k_{RS}, k_{TS}, and L') may be determined. As in the conventional analysis of Scatchard plots, the number of binding sites per molecule of protein of known molecular weight (n) is given by the intercept with the abscissa of data obtained in nearly saturating ligand concentrations. From the ordinate intercept of a linear extrapolation of this region of the curve, the value of k_{RS} may be estimated as shown in Figure 1a. The value of the allosteric constant L' can then be estimated by comparing either the normalized ordinate of the maximum of the Scatchard plot $(n\bar{Y}/S)_{max}k_{RS}$ or the corresponding value of the abscissa, $n\bar{Y}_{max}$ with data computed according to eq 1. For a tetramer having a small conformational equilibrium constant (L' between 1 and 15) the coordinates of the Scatchard maximum are rather insensitive to the value of k_{TS} and permit the determination of L' within a narrow range as shown in Figure 1b. Using the values of n, k_{RS}, and L' obtained in this way, one may calculate the ratio of substrate affinities for the two states $c = k_{RS}/k_{TS}$ from the ordinate intercept of binding data obtained at low ligand concentrations. Since the resultant values of L' and c depend on the initial estimate of k_{RS} (which becomes increasingly uncertain for systems with high values of L'), this method is used only to obtain a range of values of L' and c which are consistent with each other and with the highest and lowest estimates of k_{RS}. The uncertainty in these parameters is then reduced by making additional comparisons of the observed values of the free ligand concentration corresponding to half-saturation of the sites $S_{1/2}$ and the maximum slope of the Hill plot $n_{H\,max}$ with data computed according to eq 1 (see Rubin and Changeux, 1966). The final choice of a compatible set of parameters within this restricted range is based on visual agreement between empirical and computed saturation functions and Scatchard plots.

$$\mathrm{Lim}\left(\frac{n\bar{Y}}{S}\right)_{\bar{Y}\to 0} = \frac{n(L'c+1)}{k_{RS}(L'+1)} \qquad (7)$$

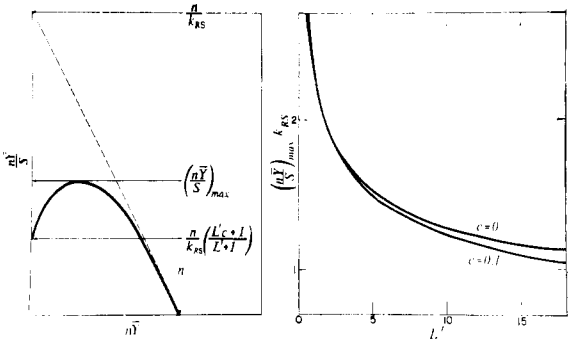

FIGURE 1: Evaluation of the parameters of the model of Monod *et al.* from the Scatchard plot of ligand binding data. (a) Left: properties of the Scatchard plot computed according to eq 1 for $n = 4$, $L' = 10$, $c = 0$. (b) Right: dependence on L' of the normalized ordinate of the maximum of the Scatchard plot $(n\bar{Y}/S)_{max}k_{RS}$, computed from eq 1 for $n = 4$, $c = 0$, and c = 0.1.

Unlike the vividly cooperative interactions of ATCase with its substrate, the binding of the feedback inhibitor or its derivative BrCTP is characterized by a linear Scatchard plot within the accuracy of the experimental data. From these results, the number of binding sites for the inhibitor and an approximate value for its intrinsic dissociation constant from the protein state with higher affinity k_{TI} can be obtained by the conventional analysis of the Scatchard plot. The dissociation constant from the protein state with weaker affinity k_{RI} or the affinity ratio $d = k_{RI}/k_{TI}$, however, cannot be determined by this method. The relative affinity of BrCTP for the putative conformational states of ATCase was therefore ascertained indirectly from the conformational studies of the enzyme in the presence of the inhibitor, as described in the next section.

Methods of Analysis of Conformational Data. RATE OF PMB FIXATION. The main assumptions involved in the analysis of data for the reactivity of ATCase toward PMB in terms of the model of Monod *et al.* are: (1) that the measured pseudo-first-order rate constant of mercurial fixation (g) may be identified with the number-average value of the intrinsic rate constants of enzyme molecules in the two conformational states R and T, and (2) that the intrinsic rate constants g_R and g_T are independent of the interactions

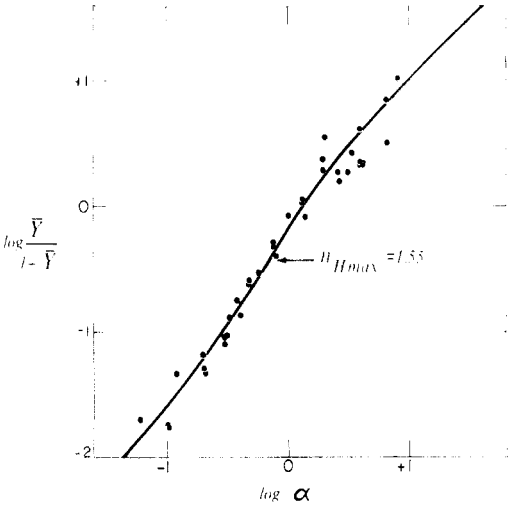

FIGURE 2: Hill plot of the fractional saturation ($\bar{Y}$) of native ATCase by succinate. Data from paper I normalized to values of $\alpha = [\text{Succ}]/k_{RS}$ using $k_{RS} = 4.75 \times 10^{-4}$ M. Solid line: theoretical curve computed from eq 1 for $n = 4$, $L = 4$, and $c \leq 10^{-3}$. The maximum slope $n_{H\,max} = 1.55$.

of the enzyme with the allosteric effectors. Variations of the observed rate constant (g) with substrate and/or inhibitor concentration are then attributed entirely to shifts in the conformational equilibrium, expressed in terms of the state function $\bar{R}$ (eq 8). The intrinsic

$$g = \bar{R}g_R + (1 - \bar{R})g_T = \bar{R}(g_R - g_T) + g_T \quad (8)$$

rates g_R and g_T are determined from the rates of PMB fixation measured in the absence of succinate, $g(\alpha = 0)$, and in the presence of saturating concentrations, $g(\alpha \rightarrow \infty)$. In particular, g_R is identified with the rate measured at saturating succinate concentrations or with the extrapolated rate at infinite ligand concentration obtained from a double-reciprocal plot. This approximation, that $g_R = g(\alpha \rightarrow \infty)$, is valid so long as $\bar{R}(\alpha \rightarrow \infty)$ is close to unity, in other words, when $L'c^n$ is negligible (eq 4). From the detailed analysis of the succinate binding data, $L'c^n$ is less than 4×10^{-3}; the assumption is therefore justified for ATCase under these conditions. The intrinsic rate of mercurial binding to the T state cannot be measured directly, but may be evaluated from $g(\alpha = 0)$, $g(\alpha \rightarrow \infty)$, and the numerical value of L determined from the equilibrium dialysis data as described above. The

$$g_T = [g(\alpha \longrightarrow \infty) - g(\alpha = 0)](L + 1)/L \quad (9)$$

experimental data for the rate of mercurial binding are then related to the theroetical state function $\bar{R}$ according to eq 8.

Determination of d, the ratio of the microscopic dissociation constants of BrCTP from the R and T states, is based on similar experiments done in the presence of the inhibitor analog. The rate of PMB fixation measured in the presence of saturating BrCTP in the absence of succinate, $g(\alpha = 0, \beta \rightarrow \infty)$, corresponds to the lower limit of the state function given in eq 5. The ratio $d = k_{RI}/k_{TI}$ may therefore be calculated from $g(\alpha = 0, \beta \rightarrow \infty)$ according to eq 5 and 8 and the values of g_R and g_T previously determined in the experiments with succinate. An independent estimate of d is obtained from mercurial binding experiments performed in the presence of both the substrate and inhibitor analogs. The values of $\bar{R}$ are first plotted as a function of BrCTP for several fixed concentrations of succinate, as shown in Figure 6. The initial and plateau values of these curves are then replotted as a function of succinate concentration, as shown in Figure 7. The value of d is determined from the normalized succinate concentration $\alpha_{\bar{R}/2}$ at which the state function in saturating inhibitor equals 0.5.

$$d = L^{-1/n}\left(\frac{1 + \alpha_{\bar{R}/2}}{1 + \alpha_{\bar{R}/2}c}\right) \quad (10)$$

SEDIMENTATION VELOCITY EXPERIMENTS. A similar analysis in terms of the model of Monod *et al.* is applied to the results of Gerhart and Schachman (1968) for the variation of the sedimentation coefficient of ATCase as a function of succinate concentration. The available data in this case are identified with the difference in the number-average sedimentation coefficient (s) in the absence of succinate and in the presence of a fixed concentration of this compound.

$$\Delta s(\alpha) = s(\alpha) - s(\alpha = 0) = (s_R - s_T)[\bar{R}(\alpha) - \bar{R}(\alpha = 0)] \quad (11)$$

where $\alpha = [\text{Succ}]/k_{RS}$. The ratio $\Delta s(\alpha)/\Delta s(\alpha \rightarrow \infty)$ is clearly independent of the intrinsic sedimentation coefficients of the respective states, s_R and s_T, and may be compared directly with the relative state function $\bar{R}r$ given in eq 6. Finally, the values of the relative

$$\frac{\Delta s(\alpha)}{\Delta s(\alpha \longrightarrow \infty)} = \bar{R}r = \frac{\bar{R}(\alpha) - \bar{R}(\alpha = 0)}{\bar{R}(\alpha \longrightarrow \infty) - \bar{R}(\alpha = 0)} \quad (12)$$

state function $\bar{R}r$ obtained from the sedimentation analysis are converted to the form of the absolute state function $\bar{R}$ by means of eq 6 using the values of L' and c determined in the succinate binding experiments.

Results

Succinate Binding. NATIVE ENZYME. The equilibrium dialysis results presented in paper I demonstrate that native ATCase mediates cooperative homotropic interactions among molecules of succinate a nonreactive analog of one of the substrates aspartate, in the presence of saturating levels of the other substrate, carbamyl phosphate. As illustrated in Figures 2 and 3,

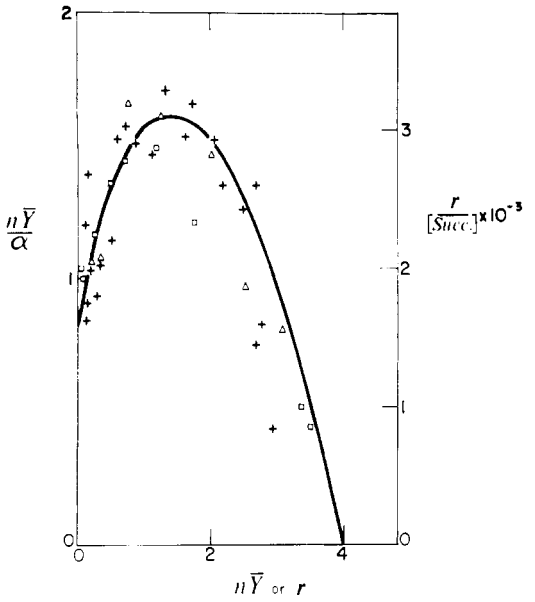

FIGURE 3: Scatchard plot of equilibrium dialysis data for binding of succinate to native ATCase (paper I). Solid line: theoretical curve computed from eq 1 for $n = 4, L = 4, c \leq 10^{-3}$, and $k_{RS} = 4.75 \times 10^{-4}$ M.

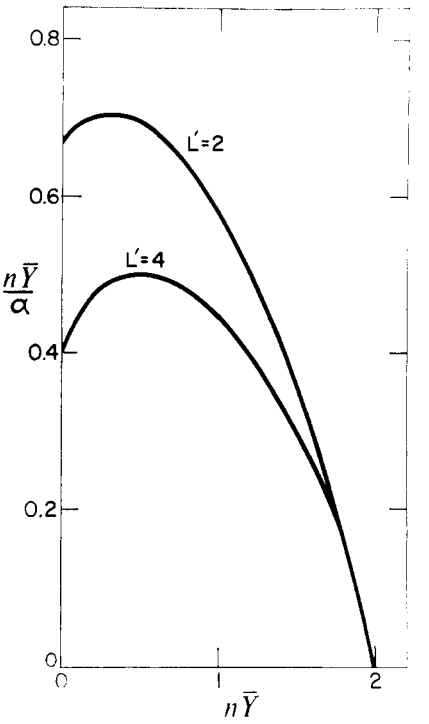

FIGURE 4: Scatchard plot of theoretical binding function computed from eq 1 for a *dimer* ($n = 2$) with $c = 0$ and $L' = 2$ or 4. Compare with Figure 1, paper I.

the succinate binding data can be described nearly quantitatively by the saturation function of the model of Monod *et al.* (eq 1) using parameters determined as described above. The Scatchard plot of the succinate binding data is first analyzed to determine the number of substrate binding sites, $n = 3.8 \pm 0.2$ per molecular weight unit of 3.0×10^5, and to estimate the microscopic dissociation constant of succinate from the R state of the protein, $k_{RS} = 5.0 \pm 0.5 \times 10^{-4}$ M. The following three characteristic points of the same experimental curve are then considered: (1) the ordinate of the maximum of the Scatchard plot

$$(n\bar{Y}/S)_{max} = 3.2 \pm 0.2 \times 10^{-3}\ \text{M}^{-1}$$

(2) the intercept with the ordinate of a linear extrapolation of the binding curve at low ligand concentrations

$$\text{Lim}\left(\frac{n\bar{Y}}{S}\right)_{\bar{Y}\to 0} = 1.6 \pm 0.15 \times 10^{-3}\ \text{M}^{-1}$$

(3) the free ligand concentration corresponding to half-saturation

$$S_{1/2} = 6.3 \pm 0.2 \times 10^{-4}\ \text{M}$$

On the basis of these estimates and the nearest integral number of succinate binding sites ($n = 4$), the following set of values of the parameters was selected:

$$k_{RS} = 4.75 \times 10^{-4}\ \text{M}$$

$$L = L' \pm 4.0$$

$$c = k_{RS}/k_{TS} \leq 10^{-3}$$

Computations based on this set of parameters and on eq 1 are compared in Figures 4 and 5 with experimental succinate binding data plotted in the coordinate systems of Hill (Brown and Hill, 1922) and Scatchard. The selected set of constants leads to good agreement between theory and experiment and indicates that under the conditions specified in paper I: (1) only one of the putative conformational states of ATCase exhibits significant affinity for succinate (exclusive binding), and (2) the spontaneous distribution of enzyme molecules between the two states favors the form with negligible affinity for succinate (state T).

Catalytic Subunit. The Scatchard plot of the equilibrium dialysis data for the binding of succinate to the catalytic subunit of ATCase is linear. The value of the dissociation constant of succinate from this subunit (5.5×10^{-4} M) is remarkably close to the value of the intrinsic constant (k_{RS}) calculated previously for the R state of the native enzyme. This observation suggests that the configuration of the active sites, and thus the conformation of the catalytic subunits, are very similar in the R state of the native enzyme and in the isolated subunit, in contrast with the conformation of the catalytic subunits in the T state, in which the substrate binding sites are impaired.

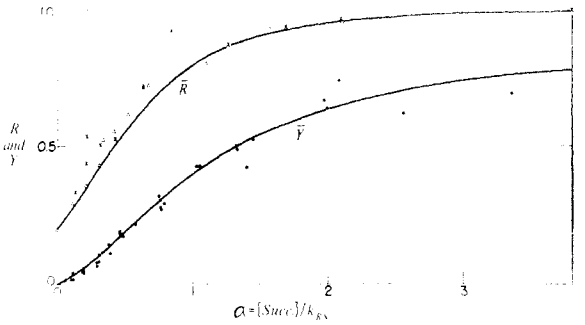

FIGURE 5: Comparison of succinate binding to native ATCase with the corresponding changes in the enzyme conformation. Equilibrium dialysis data for succinate binding (●) are from paper I. Data for the pseudo-first-order rate constant of PMB fixation (△) and for the sedimentation coefficient (X) (paper II) have been converted to values of the state function as described in the Theory section. Theoretical binding function ($\bar{R}$) and state function ($\bar{Y}$) were computed from eq 1 and 2 using as input the values of the parameters determined from the binding data: $n = 4$, $L = 4$, $k_{RS} = 4.75 \times 10^{-4}$ M, and $c \leq 10^{-3}$.

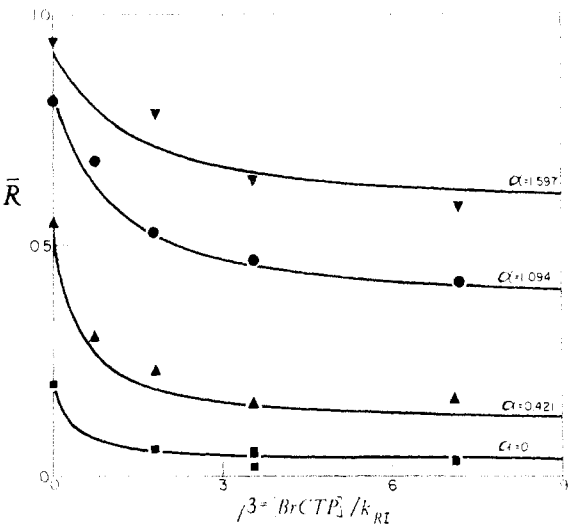

FIGURE 6: Antagonism between succinate and BrCTP followed by the reactivity of native ATCase to PMB. Experimental data from paper II converted to values of the state function $\bar{R}$ as described in Theory. Solid lines were computed from eq 2 for $n = 4$, $L = 4$, $c \leq 10^{-3}$, $d = 1.7$, $k_{RS} = 4.75 \times 10^{-4}$ M, and $k_{TI} = 1.16 \times 10^{-5}$ M for the indicated values of $\alpha = [\text{Succ}]/k_{RS}$.

A further remarkable property of the catalytic subunit is that despite the presence of *two* substrate receptors, this unit does not appear to mediate homotropic interactions between succinate molecules bound to these sites. The computed Scatchard plots in Figure 4 confirm that these interactions would be detected in an allosteric dimer having a conformational equilibrium constant identical with that estimated for the native enzyme ($L = 4$) as well as in a dimer in which $L = 2$ in accordance with the assumptions of F. H. C. Crick and J. Wyman (in preparation). The absence of experimental evidence for homotropic interactions in the isolated catalytic subunit indicates that the homotropic interactions among substrate molecules depend strictly upon the integration of the catalytic subunits and the regulatory subunits into the native oligomeric structure.

Comparison of Succinate Binding to Native ATCase with the Corresponding Changes in the Enzyme Conformation. Data presented in paper II demonstrate that the interaction of native ATCase with specific ligands is accompanied by a reorganization of the three-dimensional structure of the enzyme. This change of conformation does not seem to involve a change in the state of aggregation of the molecule in the presence of effectors and has been interpreted as an isomerization of the protein (paper II). On the basis of this assumption and the parameters deduced from the succinate binding studies, the curve representing changes in the enzyme conformation upon increasing the succinate concentration is expected to be distinct from and to precede the corresponding saturation curve. This prediction is confirmed by the respective succinate concentrations at which the empirical functions $\bar{Y}$ and $\bar{R}r$ equal 0.5; for the saturation function (paper I), the succinate concentration corresponding to midpoint is 6.3×10^{-4} M; for the relative state function (paper II), 2.0×10^{-4} M. Furthermore, using the values of n, L', k_{RS}, and k_{TS} obtained from the analysis of the succinate binding data in terms of eq 1, one can compute the corresponding theoretical state function according to eq 2. As shown in Figure 5, the data for both the sulfhydryl group reactivity and the sedimentation velocity experiments (paper II) replotted as described in the Theory section can be superimposed almost exactly on the predicted state function. The separation between the state and saturation functions observed for ATCase upon interaction with succinate is thus compatible both qualitatively and quantitatively with the predictions of the two-state model for allosteric interactions.

Effects of BrCTP and Succinate on the Enzyme Conformation. An explicit assumption of the model of Monod *et al.* is that the number of conformations accessible to an allosteric oligomer is *small* and is independent of whether the considered interactions involve identical or unlike ligand molecules. It is therefore of interest to investigate whether the structural effects of the feedback inhibitor (CTP) or its analog (BrCTP) can be accounted for quantitatively without postulating more than the two conformational states required to describe the interactions of ATCase with succinate. According to the two-state model, CTP and BrCTP which are known to inhibit the catalytic activity of the enzyme should bind preferentially to the T state for which the substrate has little affinity. Extending this reasoning to the structural

TABLE I: Antagonism of BrCTP Binding to Native ATCase by Succinate.

Succinate (M):	0	2.5×10^{-3}	5.0×10^{-3}
S/k_{RS}:	0	5.26	10.52
Total BrCTP (M)			
1.0×10^{-6}	0.0083	0.0073	0.0068
	0.0087	*0.0056*	*0.0056*
7.20×10^{-5}	0.0595	0.0522	0.0482
	0.0620	*0.0405*	*0.0404*
1.09×10^{-4}	0.704	0.578	0.533
	0.644	*0.526*	*0.525*

[a] Fractional saturation of inhibitor binding sites measured by equilibrium dialysis with BrCTP (paper I) (roman type). Fractional saturation computed according to eq 1 for $n = 4$, $L = 4$, $c = 10^{-3}$, $d = 1.7$, $k_{RS} = 4.75 \times 10^{-4}$ M, and $k_{TI} = 1.16 \times 10^{-5}$ M (italics).

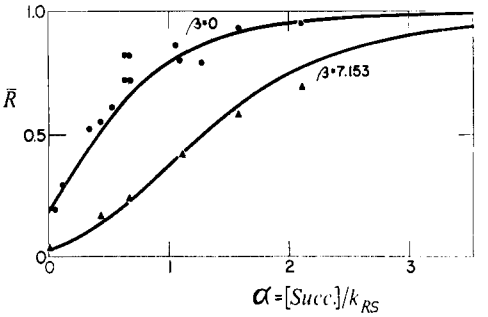

FIGURE 7: Effect of succinate on the reactivity of native ATCase to PMB in the absence (●) and in the presence (▲) of 1.4×10^{-4} M BrCTP. Experimental data from paper II were converted to values of the state function $\bar{R}$ as described in Theory. Solid lines computed from eq 2 for $n = 4$, $L = 4$, $c \leq 10^{-3}$, $d = 1.7$, $k_{RS} = 4.75 \times 10^{-4}$ M, and $k_{TI} = 1.16 \times 10^{-5}$ M.

effects of the various ligands, the reactivity of ATCase to PMB which is increased in the presence of succinate, should be decreased by the addition of the inhibitors. Using the value of the allosteric constant previously determined from the succinate binding data ($L = 4$), one calculates that the decrease in the rate of PMB binding due to BrCTP alone should not exceed 20% of the maximal increase observed in the presence of succinate. Figure 6 indicates that this prediction is fulfilled within the experimental uncertainty.

The combined effects of BrCTP and succinate on the reactivity of ATCase to PMB is similarly consistent with the two-state model. Moreover, the antagonism between the substrate and inhibitor analogs displays the same characteristic property as the antagonism observed in equilibrium dialysis experiments; namely, a large excess of BrCTP reverses only *partially* the action of succinate. A simple interpretation of this incomplete antagonism is that BrCTP has significant but unequal affinity for *both* conformational states (nonexclusive binding). Thus, even saturating levels of this compound cannot shift the isomerization equilibrium completely in either direction (Rubin and Changeux, 1966). From the characteristics of the limiting state of the equilibrium obtained in saturating BrCTP, the coefficient of nonexclusive binding of the inhibitor analog ($d = k_{RI}/k_{TI}$) is readily evaluated. By this method, the affinity of BrCTP for the T state is ascertained to be only 1.7 times its affinity for the R state, in striking contrast with the almost exclusive affinity of succinate for the latter state ($c = k_{RS}/k_{TS} \leq 10^{-3}$).

Finally, combining the value of $d = 1.7$ with the estimate of $k_{TI} = 1.16 \times 10^{-5}$ M from the BrCTP binding studies and the set of parameters derived from the succinate binding data, one may calculate according to eq 1-3 the state and saturation functions expected in the presence of any mixture of the considered ligands. Figures 6-8 and Table I demonstrate that a consistent interpretation of all the available data is obtained.

Conclusions

Data obtained by three physical chemical techniques concerning the binding and structural effects of several ligands on ATCase have been shown to be compatible with the model for allosteric interactions proposed by Monod *et al.* More precisely, a set of values can be found for the parameters of this model which lead to a coherent, quantitative description of data on the interactions of ATCase with analogs of the feedback inhibitor (BrCTP) and of one of the substrates (succinate) in the presence of saturating levels of the other substrate (carbamyl phosphate). The particular values of the constants corresponding to the experimental conditions specified in papers I and II indicate that (1) the postulated equilibrium of protein molecules between the conformational states R and T spontaneously favors in a 4:1 ratio the T state which binds the inhibitor preferentially but exhibits negligible affinity for succinate ($L = \bar{T}/\bar{R} = 4$), and (2) the free-energy change involved in the R $\rightleftharpoons$ T transition is extremely small ($\Delta F < 1$ kcal mol^{-1}).[5]

In view of the low value of the allosteric constant (L), the detection of homotropic interactions in the binding of succinate is attributed to the nearly exclusive affinity of succinate for one of the protein states.

[5] The conformational equilibrium constant ($L = e^{\Delta F/RT}$) was evaluated from succinate binding data obtained in the presence of an excess of carbamyl phosphate. The resultant low value of $L = 4$, might be due, in part, to the preferential binding of carbamyl phosphate to the same state to which succinate binds. In the absence of carbamyl phosphate, the values of L and ΔF obtained for the conformational transition might be much larger.

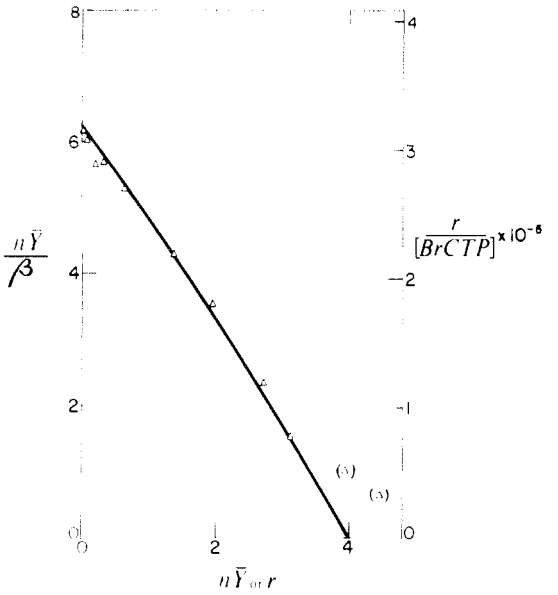

FIGURE 8: Scatchard plot of equilibrium dialysis data for [^{14}C]BrCTP binding to native ATCase in the presence of 8×10^{-4} M carbamyl phosphate. Dialysis time at 21° using the method of Myer and Schellman (1962) was 48 hr. Data for r/(BrCTP) are normalized to values of $n\bar{Y}/\beta = (nY/I)k_{RI}$ using $k_{RI} = 1.97 \times 10^{-5}$ M. Solid line: theoretical curve for inhibitor binding computed for $n = 4$, $L = 4$, $d = 1.7$, and $k_{TI} = 1.16 \times 10^{-5}$ M.

The large difference in the microscopic dissociation constants of succinate from the two states is, in turn, thought to indicate major rearrangements of the active sites during the conformational transition. Conversely, the absence of homotropic interactions for BrCTP alone and the incomplete antagonism between the inhibitor and substrate analogs suggest that BrCTP has significant but unequal affinity for both conformations of the protein and thus that the differences in the configuration of the regulatory sites in the R and T states are small. The effectiveness of the *in vivo* regulation of the activity of ATCase by its feedback inhibitor would therefore be due primarily to the dramatic differences in the affinity of the active sites for aspartate.

Although the experimental data on ATCase appear to fulfill the predictions of the model of Monod *et al.*, alternative models might also account for these results. We consider first the proposal that specific conformational changes are "induced" by and hence coincident with the binding of the various ligands (Induced Fit Theory; Koshland, 1963). This model does not include the following two postulates made by Monod *et al.* (1) The allosteric interactions of a regulatory enzyme are mediated by transitions of the protein between a small number of discrete conformational states (see also Lumry *et al.*, 1966) and (2) the conformational states are independent of the structure of any particular ligand and are present in a reversible equilibrium in the absence of ligands. The Induced Fit Theory does not simply account for the observations on ATCase that (1) both the heterotropic and homotropic interactions mediated by the enzyme can be accounted for quantitatively in terms of only two conformational states, and (2) the progressive conformational changes do not coincide with (but actually precede) the saturation of binding sites as the succinate concentration is increased.

It should be recalled, however, that the observed interactions involve ligands bound to receptors which are presumably located far apart on the enzyme surface. The present experimental data indicate that these long-range interactions responsible for the regulatory properties of the enzyme are associated with a conformational isomerization of the protein molecule. These long-range, allosteric interactions may be superimposed on short-range interactions which account for *local* effects and which might depend on the microscopic structure of the ligand and are thus "induced" by its very binding with the enzyme surface (Koshland, 1963). This latter class of interactions might include certain effects observed at the level of the catalytic sites of ATCase, such as the promotion of succinate binding by carbamyl phosphate in both native ATCase and its catalytic subunit (see paper I).

Finally, we consider a generalization of the model of Monod *et al.* in which the transitions between the conformational states of the protein are only partially coordinated with respect to the several subunits (Koshland *et al.*, 1966; S. J. Edelstein, in preparation). Such a model, which allows for the presence of unsymmetrical or "hybrid" states in which some protomers correspond to the R state and some to the T state, cannot be definitively ruled out, since a small quantity of these hypothetical "hybrid" states would remain undetected using the present experimental approach. However, the observation that the conformational alterations approach completion at concentrations of succinate which only partially saturate the binding sites of the enzyme indicates that the hybrid states, if present, are less stable than the symmetrical states and thus that the transitions of ATCase are largely concerted with respect to the several protomers.

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