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Allosteric Regulation of Aspartate Transcarbamoylase. Analysis of the Structural and Functional Behavior in Terms of a Two-State Model†

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ABSTRACT: The kinetic and physical properties of the allosteric enzyme, aspartate transcarbamoylase from *Escherichia coli,* have been analyzed according to the two-state model (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12,* 88). An internally consistent set of calculated parameters accounted quantitatively for the results from diverse experiments including: (a) enzyme kinetics as a function of the concentration of aspartate in the presence of saturating carbamoyl phosphate; (b) gross conformational changes of the enzyme, revealed by the decrease in the sedimentation coefficient and the increase in the reactivity of the sulfhydryl groups of the regulatory subunits, as a function of the extent of saturation of the active sites by the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate; (c) stimulation of enzymic activity at low concentrations of the substrate analogue, succinate; and (d) effects of the inhibitor, CTP, and the activator, ATP, on the kinetic and physical properties of the enzyme. The data were interpreted in terms of an equilibrium between a constrained or low-affinity (T) state and a relaxed or high-affinity (R) form of the enzyme and the perturbation of the equilibrium by the addition of various ligands. In the absence of any ligand the equilibrium constant, [T]/[R], was 250 indicating that the T state was 3.3 kcal/mol more stable than the R state. The affinity of the T state for aspartate, succinate, and *N*-(phosphonacetyl)-L-aspartate was at least 20 times weaker than that of the R state. A slight preferential binding of carbamoyl phosphate to the R state caused a shift of the allosteric equilibrium constant to 7. When ATP which also binds slightly better to the R state was present along with carbamoyl phosphate, the equilibrium constant was reduced to 2. In contrast, the slight preferential binding of CTP to the T state led to a shift of the equilibrium constant to 35 when carbamoyl phosphate was present and to 1250 in the absence of that substrate (with ATP and no carbamoyl phosphate the constant is 70). Although the nucleotides and the substrate, carbamoyl phosphate, bind to different polypeptide chains in the enzyme molecule, their effects on the kinetic and physical properties are additive in terms of influencing the allosteric transition. The results cited are consistent with the view that the entire enzyme molecule undergoes a concerted transition which is influenced by binding of different ligands to the various polypeptide chains in the protein.

The demonstration that aspartate transcarbamoylase (ATCase)[1] (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) from *Escherichia coli* exhibits a sigmoidal dependence of activity on substrate concentration and is subject to feedback inhibition by CTP (Gerhart and Pardee, 1962) represents one of the earliest examples of the regulation of enzyme activity. When it was shown subsequently (Gerhart and Schachman, 1965) that ATCase contained distinct catalytic and regulatory subunits, these two phenomena characteristic of allosteric enzymes, termed homotropic and heterotropic effects (Monod et al., 1965), became the subject of investigations relating structure to function (Gerhart, 1970; Jacobson and Stark, 1973a; Schachman, 1974). Both allosteric effects have been postulated to be the consequence of ligand-promoted conformational changes in the enzyme whereby the binding of ligands to the oligomeric protein affects the subsequent binding to other sites on the same molecule (Monod et al., 1965; Koshland et al., 1966).

Ligands which bind to the active sites on the catalytic subunits of ATCase lead not only to an increase in the effective hydrodynamic volume of the molecules but also to a sixfold enhancement of the chemical reactivity of the 24 sulfhydryl groups on the regulatory subunits (Gerhart and Schachman, 1968). These alterations in the properties of ATCase upon the binding of active-site ligands are indicative of a gross conformational change in the enzyme molecules and have been interpreted (Changeux and Rubin, 1968) in terms of the model proposed by Monod et al. (1965). However, at that time knowledge of the structure of ATCase was insufficient and binding data for substrate analogues were inadequate. Hence a renewed attempt was made to determine whether the two-state model could account for additional enzyme kinetic data and new results relating conformational changes to the extent of binding of the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA).

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[1] Abbreviations used are: ATCase, aspartate transcarbamoylase; PALA, *N*-(phosphonacetyl)-L-aspartate; CbmP, carbamoyl phosphate; Asp, aspartate; PMB, *p*-mercuribenzoate; n_H, Hill coefficient.

According to the model of Monod et al. (1965), the enzyme molecules are in a dynamic equilibrium between two conformational states and the ligand-promoted allosteric transition from one to the other is concerted so that the conformation of each protomer in a single molecule is the same even though some protomers may be liganded and others nonliganded. Hence in the conversion of the enzyme from the constrained to the relaxed state the extent of the conformational change need not be directly proportional to the degree of ligand saturation. As seen in the preceding papers (Howlett and Schachman, 1977; Blackburn and Schachman, 1977) both the decrease in the sedimentation coefficient and the increase in reactivity of the sulfhydryl groups of ATCase were complete even though a substantial fraction of the active sites was still unsaturated. In this paper these experimental results were analyzed quantitatively according to the two-state theory with internally consistent parameters describing the ligand binding to each state and the equilibrium between the two conformational states. The parameters evaluated from the enzyme kinetics at varying aspartate concentration and the physical chemical experiments were used to interpret the activation of ATCase caused by the substrate analogue, succinate, which competes with aspartate for binding at the active sites. In addition the same parameters were employed to analyze the enzyme kinetics at a fixed aspartate concentration and increasing amounts of the first substrate, carbamoyl phosphate. As shown in this paper the two-state model accounts quantitatively for a great variety of experimental results and provides a unifying framework for analyzing the allosteric properties of ATCase.

Theory

Ordered Ligand Binding in the Two-State Model. Since the binding of substrates (and analogues) to ATCase is ordered (Wedler and Gasser, 1974; Jacobson and Stark, 1975) with carbamoyl phosphate (CbmP) binding prior to aspartate (or succinate), it was necessary to extend the treatment of Monod et al. (1965) to encompass enzymes with two substrates and ordered binding.[2]

Consider the enzyme existing either in the high-affinity (relaxed) form, designated R, or in the low-affinity (taut or constrained) conformation, designated T. Both forms can bind the substrate, CbmP, with the intrinsic affinities described by the dissociation constants, $K_{R(CbmP)}$ and $K_{T(CbmP)}$, for the enzyme-substrate complexes in the R and T states, respectively. Unless there is binding of CbmP to the enzyme, neither form can bind the second substrate, aspartate (Asp). Hence the various species present at equilibrium are $R(CbmP)_i(Asp)_j$ and $T(CbmP)_i(Asp)_j$, where i varies from 0 to n, j varies from 0 to i, and n is the number of active sites on the enzyme. The concentrations of these species are given by

$$R(CbmP)_i(Asp)_j = F_{n,i}F_{i,j}R\beta^i\alpha^j \qquad (1a)$$

and

$$T(CbmP)_i(Asp)_j = F_{n,i}F_{i,j}T\beta^i b^i_{CbmP}\alpha^j c^j_{Asp} \qquad i = 0, n; j = 0, i \qquad (1b)$$

where

$$\beta = [CbmP]/K_{R(CbmP)},\ \alpha = [Asp]/K_{R(Asp)},\ b_{CbmP} = K_{R(CbmP)}/K_{T(CbmP)},\ c_{Asp} = K_{R(Asp)}/K_{T(Asp)}$$

and $F_{n,i}$ and $F_{i,j}$ are statistical factors given by

$$F_{x,y} = x!/y!(x-y)! \qquad (2)$$

The fraction of sites on the protein, $\bar{Y}_{Asp}$, occupied by Asp is described by the binding function

$$\bar{Y}_{Asp} = \frac{\sum_{i=0}^{n}\sum_{j=0}^{i} jF_{n,i}F_{i,j}\beta^i\alpha^j(1 + Lb^i_{CbmP}c^j_{Asp})}{n\sum_{i=0}^{n}\sum_{j=0}^{i} F_{n,i}F_{i,j}\beta^i\alpha^j(1 + Lb^i_{CbmP}c^j_{Asp})} \qquad (3)$$

where $L = [T]/[R]$. By use of the binomial theorem eq 3 reduces to

$$\bar{Y}_{Asp} = \alpha\beta(1 + \beta(1+\alpha))^{n-1} + \frac{L\alpha c_{Asp}\beta b_{CbmP}(1 + \beta b_{CbmP}(1 + \alpha c_{Asp}))^{n-1}}{(1 + \beta(1+\alpha))^n + L(1 + \beta b_{CbmP}(1 + \alpha c_{Asp}))^n} \qquad (4)$$

Equation 4 describes the dependence of bound Asp as a function of the concentrations of both CbmP and Asp. A state function, $\bar{R}$, describing the fraction of enzyme molecules in the R state at various substrate levels can also be written. This is given by

$$\bar{R} = \frac{(1 + \beta(1+\alpha))^n}{(1 + \beta(1+\alpha))^n + L(1 + \beta b_{CbmP}(1 + \alpha c_{Asp}))^n} \qquad (5)$$

At saturating concentrations of the first substrate, CbmP, β becomes large, and eq 4 and 5 reduce to the well-known equations of Monod et al. (1965)

$$\bar{Y}_{Asp} = \frac{\alpha(1+\alpha)^{n-1} + L'\alpha c_{Asp}(1 + \alpha c_{Asp})^{n-1}}{(1+\alpha)^n + L'(1 + \alpha c_{Asp})^n} \qquad (6)$$

$$\bar{R} = \frac{(1+\alpha)^n}{(1+\alpha)^n + L'(1 + \alpha c_{Asp})^n} \qquad (7a)$$

where

$$L' = Lb^n_{CbmP} \qquad (7b)$$

In this treatment L represents the intrinsic conformational equilibrium constant for the two states of the enzyme in the absence of any substrates and L' is the analogous value for the enzyme in the presence of the first substrate, CbmP.

The limiting values of $\bar{Y}_{Asp}$ in the presence of saturating CbmP are zero and unity. In contrast $\bar{R}$ under similar conditions varies from

$$1/(L'+1) \leq \bar{R} \leq 1/(L'c^n_{Asp} + 1) \qquad (8)$$

For finite values of L' the lower limit of $\bar{R}$ is greater than zero and the upper limit is unity for $c_{Asp} = 0$. Hence, Changeux and Rubin (1968) introduced the relative state function, $\bar{R}_r$, which varied from zero in the absence of ligands to unity when ligands were present at saturating concentration. With this function, $\bar{R}_r$, defined as $[\bar{R}(\alpha) - \bar{R}(\alpha = 0)]/[\bar{R}(\alpha \to \infty) - \bar{R}(\alpha = 0)]$, eq 7a becomes

$$\bar{R}_r = \frac{(L'c^n_{Asp} + 1)[(1+\alpha)^n - (1 + \alpha c_{Asp})^n]}{(1 - c^n_{Asp})[(1+\alpha)^n + L'(1 + \alpha c_{Asp})^n]} \qquad (9)$$

Equation 9 is particularly useful for relating the binding and state functions (an analogous form of $\bar{R}_r$ can be written from the general state function, eq 5).

Experimental Section

Materials and Methods

Solutions of ATCase, appropriate ligands, and PMB were

[2] It should be noted that there is not complete agreement that the binding is ordered (Heyde et al., 1973; Heyde, 1976). If the binding of substrates is random the initial formulation would be different, but the resulting analysis of most of the experiments would be similar to that presented here and the conclusions unaltered.

prepared as described by Howlett and Schachman (1977) and Blackburn and Schachman (1977). Enzyme assays were performed according to the procedure of Porter et al. (1969) for the determination of the effect of aspartate concentration and by the method of Davies et al. (1970) for the effect of CbmP concentration and the activation by succinate. [^{14}C]CbmP was purchased from New England Nuclear and used without further purification; more than 95% could be converted enzymically to carbamoyl aspartate and approximately 97% of the [^{14}C]CbmP was acid labile. One unit of enzyme activity corresponds to 1 μmol of carbamoyl aspartate formed per h.

Treatment of Experimental Data

Enzyme Kinetics at Varying Aspartate Concentration. Data from kinetic experiments at fixed CbmP and varying aspartate concentrations were analyzed in order to determine values of V_{max}, $K_{R(Asp)}$, L', and c_{Asp}. A value of V_{max} was obtained from Eadie plots (1942) of specific activity/[aspartate] vs. specific activity. With this value of V_{max}, a Hill plot (Brown and Hill, 1922) of the experimental data was constructed, and a value of $K_{R(Asp)}$ was obtained by extrapolation of the data at high [aspartate] to give the concentration corresponding to zero for $\log[v/(V_{max} - v)]$. The data as v/V_{max} vs. [aspartate] were then compared with theoretical curves of $\bar{Y}_{Asp}$ vs. $\alpha K_{R(Asp)}$ calculated with $n = 6$ and various values of the parameters, L' and c_{Asp}, according to eq 6. It was assumed that the relative velocity, v/V_{max}, was equivalent to the fractional saturation of active sites. Since the enzyme concentration was so low (about 10^{-9} M), virtually all of the added aspartate was free; thus $\alpha K_{R(Asp)}$ was equal to the total [aspartate]. The fitting procedure involved the use of Eadie plots and variations in L', c_{Asp}, and small adjustments in V_{max} and $K_{R(Asp)}$ until a suitable set of parameters was obtained.

Ligand-Promoted Changes in the Sedimentation Coefficient. Results from difference sedimentation experiments, $\Delta s/s$, were corrected for the buoyant density contribution of the bound ligand (Howlett and Schachman, 1977) and then normalized relative to the maximum value of $(\Delta s/s)_{corr}$ to give the percent change in the state function, $\bar{R}_r$.

The sedimentation data for the effect of PALA in the presence of CbmP were treated by using the value of L' obtained from the enzyme kinetics at saturating CbmP and varying aspartate. However, this value of L' could not be used directly in eqs 6 and 7a as in the kinetic studies since these equations were appropriate only when the second ligand (aspartate) was bound to sites already saturated by the first ligand (CbmP). Instead another set of equations was employed to account for the competition between PALA and CbmP at the same sites. This formulation is similar to that of Gregory and Wilson (1971) and Gibson (1976) and accounts for the displacement of CbmP from the active sites as PALA is bound. For this type of system eq 6 and 7a become

$$\bar{Y}_{PALA} = \gamma(1 + \beta + \gamma)^{n-1} + \frac{L\gamma d_{PALA}(1 + \beta b_{CbmP} + \gamma d_{PALA})^{n-1}}{(1 + \beta + \gamma)^n + L(1 + \beta b_{CbmP} + \gamma d_{PALA})^n} \quad (10)$$

and

$$\bar{R} = \frac{(1 + \beta + \gamma)^n}{(1 + \beta + \gamma)^n + L(1 + \beta b_{CbmP} + \gamma d_{PALA})^n} \quad (11)$$

where $\gamma = [PALA]/K_{R(PALA)}$, $d_{PALA} = K_{R(PALA)}/K_{T(PALA)}$ and the terms, β and b_{CbmP}, are those used in eq 1. If [CbmP] is in large excess relative to the concentration of active sites and $\beta \gg 1$ (also $\beta b_{CbmP} \gg 1$), we can simplify these equations to yield

$$\bar{Y}_{PALA} = \frac{\gamma'(1 + \gamma')^{n-1} + L'd'_{PALA}\gamma'(1 + \gamma' d'_{PALA})^{n-1}}{(1 + \gamma')^n + L'(1 + \gamma' d'_{PALA})^n} \quad (12)$$

and

$$\bar{R} = \frac{(1 + \gamma')^n}{(1 + \gamma')^n + L'(1 + \gamma' d'_{PALA})^n} \quad (13)$$

where

$$\gamma' = \gamma/\beta,\ d'_{PALA} = d_{PALA}/b_{CbmP},\ \text{and}\ L' = Lb^n_{CbmP}$$

Since ATCase bound PALA so tightly (dissociation constant about 10^{-8} M) virtually all of the PALA added to the enzyme at 3 mg/mL was bound up to a molar ratio of 6 even in the presence of CbmP (Collins and Stark, 1971; Jacobson and Stark, 1973b). Hence $\bar{Y}_{PALA}$ was known for each mixture of ATCase and PALA, but neither the free PALA concentration nor an accurate value of $K_{R(PALA)}$ could be determined. Thus a curve-fitting procedure was used to circumvent this lack of knowledge of γ'. Calculations of $\bar{Y}_{PALA}$ vs. γ' were made according to eq 12 with different assumed values of L' and d'_{PALA}. In an analogous way, $\bar{R}_r$ vs. γ' was calculated with the same parameters. Then theoretical curves were constructed relating $\bar{R}_r$ and $\bar{Y}_{PALA}$ for each value of L' and d'_{PALA}. These curves were then compared with the experimental data and the best-fitting value of d'_{PALA} was obtained.

The sedimentation data for solutions containing no CbmP were then analyzed so as to obtain the best value of b_{CbmP}; this was accomplished using $L = L'/b^n_{CbmP}$ and $d_{PALA} = d'_{PALA} \cdot b_{CbmP}$ and with eq 10 and 11 (with $\beta = 0$). In this way the effect of PALA on the sedimentation coefficient of the enzyme in the absence of any other ligands was used to determine the allosteric equilibrium constant, L.

Reactivity of Sulfhydryl Groups as a Function of Ligand Concentration. Experimental data for the pseudo-first-order rate constant, k, for the reaction of ATCase with PMB at varying concentrations of succinate and CbmP were analyzed as follows. First the data were normalized by obtaining the fractional change in reactivity, $(k - k_0)/(k_{max} - k_0)$, as a function of ligand concentration (k_0 and k_{max} represent, respectively, the rate constants in the absence of ligands and in the presence of saturating amounts). Then they were compared with the theoretical curves of $\bar{R}_r$ vs. $\alpha K_{R(Succ)}$ and of $\bar{R}_r$ vs. $\beta K_{R(CbmP)}$ calculated with the appropriate parameters.[3]

Activation of ATCase by Succinate. The activation of the enzyme at fixed substrate concentrations and varying amounts of the competing ligand, succinate, was analyzed by the procedure of Gregory and Wilson (1971) according to a formulation analogous to eq 10 (with succinate and aspartate competing for the same sites). In addition the data were analyzed in a more general manner with a modified form of eq 4 containing additional terms for succinate.

Enzyme Kinetics at Varying CbmP Concentration. The experimental data, v/V_{max} vs. [CbmP] at a fixed concentration of aspartate, were compared with theoretical curves of the form $\bar{Y}_{Asp}$ vs. $\beta K_{R(CbmP)}$. These curves were calculated from eq 4 with values of L, c_{Asp}, b_{CbmP}, and $K_{R(Asp)}$ determined from the sedimentation velocity studies and the kinetic experiments at

[3] $K_{R(Succ)}$ represents the dissociation constant of succinate from the R state of the enzyme–CbmP complex. It is assumed that succinate binds competitively with aspartate; hence the relative affinities of the R and T states of the enzyme for succinate can be expressed by c_{Succ} which equals $K_{R(Succ)}/K_{T(Succ)}$. These terms appear in the equations for $\bar{R}$ used to analyze the effect of succinate on the physical properties. Analogous terms are included in the equations for $\bar{Y}_{Asp}$ when succinate and aspartate are present together.

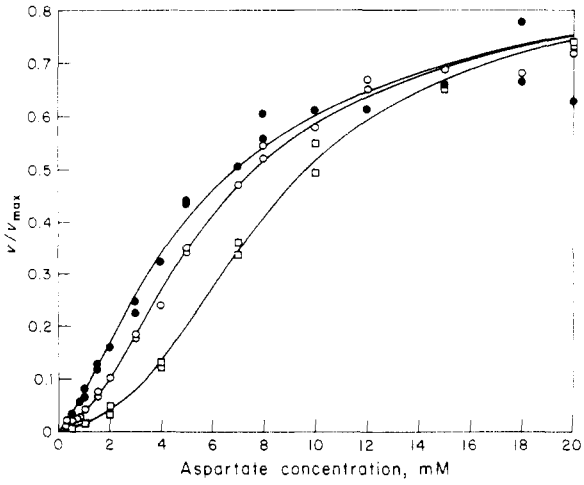

FIGURE 1: Analysis of the kinetics of ATCase (0.8 μg/mL) at varying aspartate concentration and 4 mM CbmP. The activity, v/V_{max}, was measured at 30 ± 0.2 °C with L-[^{14}C]aspartate by the method of Porter et al. (1969) with a 40 mM potassium phosphate buffer at pH 7.0 ± 0.02 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Experiments with no other ligands present are designated by ○; in the presence of 2 mM ATP by ●; and with 0.5 mM CTP by □. The curves through the experimental data were calculated theoretically ($\bar{Y}_{Asp}$ vs. $\alpha K_{R(Asp)}$) with the parameters listed in Table I.

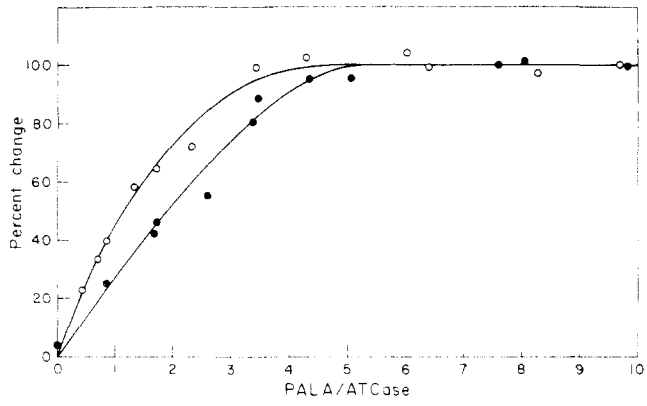

FIGURE 2: Analysis of the PALA-promoted conformational change in ATCase. The fractional change in sedimentation coefficient (at 3 mg/mL and 20 ± 2.0 °C) in terms of the percent of the maximal change was measured as a function of the molar ratio of PALA to ATCase in 40 mM potassium phosphate buffer at pH 7.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA (Howlett and Schachman, 1977). Experiments without additional ligands are represented by ●; those containing 1 mM CbmP by ○. The curves were calculated theoretically ($\bar{R}_r$ vs. $n\bar{Y}_{PALA}$) with the parameters listed in Table I.

TABLE I: Parameters Obtained from Analyzing the Structural and Functional Behavior of ATCase in Terms of a Two-State Model.[a]

Parameter	Nucleotides None	ATP[b]	CTP[c]
$K_{R(Asp)}$ (mM)[d]	6.5	6.5	6.5
c_{Asp}	0.05[e]	0.05[e]	0.05[e]
c_{Succ}	0.05	0.05	0.05
d_{PALA}	0.028	0.028	0.028
b_{CbmP}	0.55	0.55	0.55
L'	7[e]	2[e]	35[e]
L	250	70	1250
$\Delta G'$ (kcal/mol)[f]	−1.2	−0.4	−2.1
ΔG (kcal/mol)[f]	−3.3	−2.5	−4.2

[a] Experimental data were obtained with solutions of ATCase in 40 mM phosphate buffer at pH 7.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Enzyme kinetics were conducted at 30 °C and physical chemical measurements at 20 °C. The parameters were calculated on the assumption that there were 6 equiv binding sites for each of the ligands, CbmP, aspartate, succinate, and PALA. [b] 2 mM. [c] 0.5 mM. [d] Values correspond to 30 °C. [e] These values were obtained at both 30 and 20 °C (see footnote 4). [f] $\Delta G'$ and ΔG are the free energy changes at 25 °C for the allosteric transition of the enzyme in the presence and absence of CbmP.

varying aspartate concentrations. Initial estimates of $K_{R(CbmP)}$ were obtained from the concentration of CbmP corresponding to half-maximal velocity. Small adjustments were then made in the values of $K_{R(CbmP)}$ and V_{max} to produce the best-fitting theoretical curves.

Results

Enzyme Kinetics at Varying Aspartate Concentration. Figure 1 shows the activity of ATCase (v/V_{max}) at 30 ± 0.2 °C as a function of the concentration of aspartate at saturating CbmP (4 mM). Analysis of the sigmoidal curves first observed by Gerhart and Pardee (1962) gave a Hill coefficient (n_H) of 1.7 for ATCase in the absence of any nucleotide. In the presence of CTP (0.5 mM), n_H increased to 2.0 and the enzyme was inhibited markedly at low aspartate concentration. Conversely n_H decreased to 1.3 and the enzyme was activated upon the addition of ATP (2 mM).

The curves in Figure 1 were calculated theoretically with maximal velocities as 10.5 units/μg for solutions containing no nucleotides and as 9.0 units/μg when ATP or CTP was present. This small decrease in V_{max} is attributable to the competition of the nucleotides with CbmP at the active sites (Kleppe, 1966; Porter et al., 1969). Table I summarizes the values of $K_{R(Asp)}$, c_{Asp}, and L' used in the curve fitting.[4] The isomerization constant, L', describing the equilibrium between the constrained and relaxed states, was 7 when no nucleotides were present. In the presence of ATP or CTP, respectively, L' was 2 or 35. Hence the analysis indicates that the R state is favored by ATP and the T state by CTP. The corresponding values for the difference in free energy (at 25 °C) between the T and R states ($\Delta G'$) are given in Table I.

Ligand-Promoted Changes in Sedimentation Coefficient. Figure 2 shows the normalized changes in the sedimentation coefficient of ATCase caused by PALA both in the presence and absence of CbmP (Howlett and Schachman, 1977). The data for solutions containing the substrate were fit by a theoretical curve with $L' = 7$ and $d'_{PALA} = 0.05$. As seen in Figure 2 the sedimentation data for solutions without CbmP could then be accounted for theoretically with a value of 0.55 for

[4] The experiments cited here on enzyme kinetics were performed at 30 ± 0.2 °C in order to permit comparison with earlier literature. In addition a limited number of experiments were done at 20 ± 0.2 °C. Although V_{max} and the aspartate concentration corresponding to $0.5V_{max}$ differed for the two temperatures the parameters, n_H, c_{Asp}, and L' (with and without CTP and ATP) were identical within experimental error and the precision of the curve fitting. The sedimentation experiments were performed at 20 ± 2 °C, but one set was also performed at 30 ± 2 °C; the normalized percent change in sedimentation coefficient as a function of PALA concentration was the same at 30 as 20 °C. Unless otherwise noted all experiments were performed with a 40 mM phosphate buffer at pH 7.0 ± 0.02 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. This buffer was chosen to eliminate the aggregation of ATCase in 50 mM imidazole-acetate buffer at pH 7.0 (Howlett and Schachman, 1977). Enzyme kinetics with the imidazole-acetate buffer yielded a higher V_{max} than in phosphate but the values of n_H and L' were the same. The best-fitting value of c_{Asp} for the imidazole solutions was 0.001 compared with 0.05 obtained with phosphate buffer.

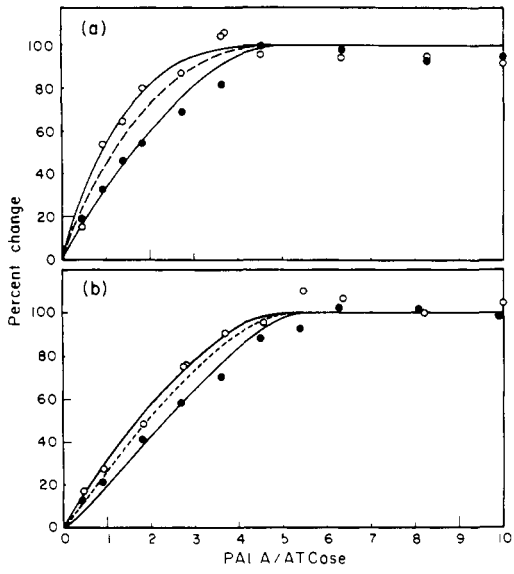

FIGURE 3: Analysis of the effect of nucleotides on the PALA-promoted conformational change in ATCase in the presence and absence of CbmP. The experimental conditions were those described in Figure 2 with ATP, ○, and CTP, ●, at concentrations of 2 mM and 0.5 mM, respectively. Experiments with 1 mM CbmP represented in a and those in the absence of the ligand in b. The theoretical curves were computed with the parameters listed in Table I. The dashed curves included in a and b are theoretical curves from Figure 2 for the PALA-promoted conformational change in the presence and absence of CbmP, respectively.

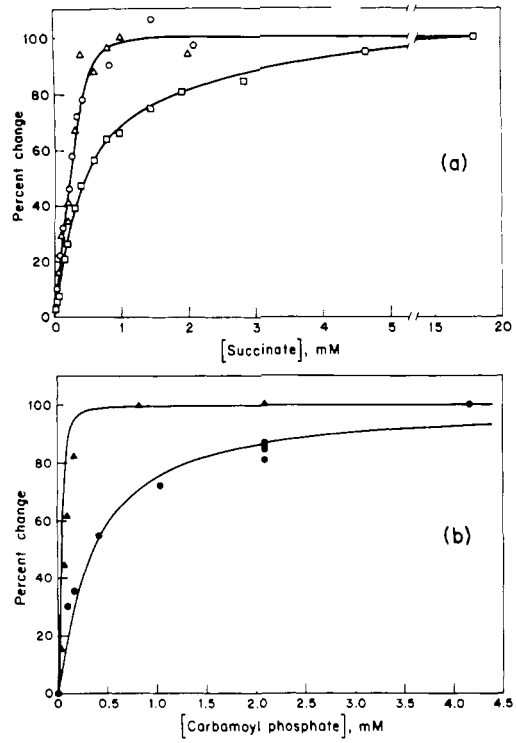

FIGURE 4: Effect of succinate and CbmP on the reaction of ATCase with PMB. Reactions were performed at 20 ± 0.2 °C with a 40 mM potassium phosphate–25 mM Tris-HCl buffer at pH 7.0 ± 0.02. The concentrations of ATCase and mercurial were 0.27 mg/mL and 0.2 mM, respectively. The pseudo-first-order rate constants were obtained as described by Blackburn and Schachman (1977). (a) Titration of the percent change in the rate constant as a function of succinate concentration in the presence of 2.1 mM CbmP is represented by ○ and the corresponding percent changes in the sedimentation coefficient of ATCase (Gerhart and Schachman, 1968) are represented by △. Measurements of the difference spectra as a function of succinate concentration are indicated by □. These latter data represent the difference between the peak at 289 nm and the trough at 286 nm. (b) Titration of the percent change in pseudo-first-order rate constant as a function of CbmP concentration. Experiments in which no other ligands were present are designated by ● and those in the presence of 1.5 mM succinate are represented by ▲. With the exception of the curve for □, in a, the curves were calculated theoretically with the parameters listed in Table I and in Results.

b_{CbmP}, which indicates that the R state binds CbmP almost twice as tightly as the T state. The intrinsic allosteric equilibrium constant, L, was calculated to be about 250; thus the physical chemical data coupled with the enzyme kinetics at varying aspartate concentration show that CbmP has a marked effect on the allosteric equilibrium (the isomerization constant was reduced from 250 to 7). The affinity of the R state for PALA was about 36-fold higher than that of the T state ($d_{PALA} = 0.028$).

The experimental data and theoretical curves for analogous studies of the PALA-promoted changes in the sedimentation coefficient of ATCase in the presence of ATP or CTP are shown in Figure 3. For the experiments with solutions containing CbmP the theoretical curves in Figure 3a were calculated with 0.05 for d'_{PALA} and L' of 2 and 35 for ATP and CTP, respectively (the values of L' obtained from the enzyme kinetics). The effects of ATP and CTP in facilitating and opposing the PALA-promoted conformational change in ATCase are illustrated in Figure 3a by the comparison with the theoretical curve (dashed, from Figure 2) for CbmP solutions without nucleotides. As seen in Figure 3b, omission of the CbmP caused a shift of all three curves so that additional PALA was needed to promote the conformational change. Nonetheless, good fits of the data were obtained with the L' values determined from the enzyme kinetics in the presence of nucleotides and the previously evaluated 0.55 for b_{CbmP}. Thus for the solutions containing no CbmP, L was shifted from 250 to 70 and 1250 by the addition of ATP and CTP, respectively. The calculated values of ΔG (at 25 °C) corresponding to these values of L are summarized in Table I.

Effect of Succinate and Carbamoyl Phosphate on the Reaction of ATCase with p-Mercuribenzoate. As seen in Figure 4a the percent increase in the pseudo-first-order rate constant for the reaction between ATCase and excess PMB varied with succinate concentration in the presence of CbmP in an identical fashion to the percent decrease in sedimentation coefficient (Gerhart and Schachman, 1968). Both of these changes occur at a lower succinate concentration than that required (cf. Figure 4a) to promote equivalent changes in the ultraviolet difference spectrum which occur upon binding of succinate at the active sites (Collins and Stark, 1969; Kirschner and Schachman, 1973a,b). The gross conformational changes indicated by the increased reactivity of the cysteinyl groups and the decreased sedimentation coefficient are readily accounted for by the theoretical curve in Figure 4a computed with $L' = 7$ and $c_{Succ} = 0.05$. For the curve fitting, a value of 0.5 mM was used for $K_{R(Succ)}$, the dissociation constant of succinate from the R conformation of the enzyme-CbmP complex. Equilibrium dialysis experiments on the enzyme in the presence of CbmP and varying amounts of succinate gave the same value (Changeux et al., 1968; Changeux and Rubin, 1968).

Analogous results for the effect of CbmP on the pseudo-first-order rate constant for the reaction of ATCase with PMB are shown in Figure 4b along with theoretical curves. The good fits of the theoretical curves (based on the parameters listed in Table I and 0.07 mM for $K_{R(CbmP)}$) show that the increase in reactivity of the sulfhydryl groups of ATCase upon the addition of CbmP can be accounted for by the same conforma-

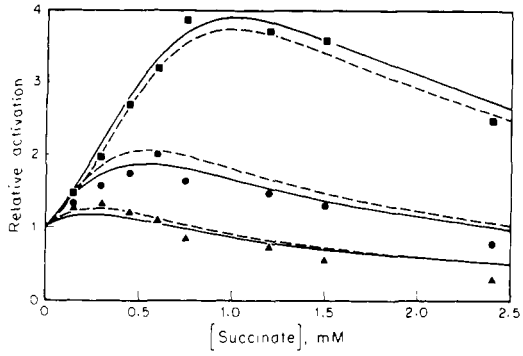

FIGURE 5: Activation of ATCase (1 μg/mL) by succinate at low concentration of aspartate (1 mM). The kinetic measurements were performed with 2 mM CbmP at 30 ± 0.2 °C by the method of Davies et al. (1970) with a 40 mM potassium phosphate buffer at pH 7.0 ± 0.02 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Results are expressed as velocity of production of carbamoyl aspartate in the presence of succinate relative to that in the absence of succinate. Experiments without other ligands are designated by ●; those with ATP (2 mM) present by ▲, and those with CTP (0.5 mM) by ■. Two sets of theoretical curves are given with those indicated by — based on eq 6 and those designated by - - - calculated from eq 4; both equations were modified to include additional terms for succinate.

tional transition of the enzyme as that observed upon the addition of succinate (with CbmP present).[5]

Activation of ATCase by Succinate. One of the striking features of the kinetic behavior of ATCase was the demonstration that its enzymic activity increased upon the addition of low concentrations of substrate analogues like maleate or succinate (Gerhart and Pardee, 1963, 1964). This stimulation of activity by compounds which compete with substrates at the active site represented one of the earliest clues that partial saturation of the enzyme led to a conformational transition resulting in enhanced substrate affinity at the unoccupied sites (Gerhart and Pardee, 1963, 1964; Gregory and Wilson, 1971; Collins and Stark, 1971; Jacobson and Stark, 1975). Figure 5 shows the relative activation of ATCase at various concentrations of succinate and a low concentration of aspartate (1 mM). CTP (0.5 mM) enhanced the relative stimulation by succinate whereas ATP (2 mM) decreased it. The experimental results are accounted for satisfactorily by the solid theoretical curves based on saturating CbmP ($\beta \gg 1$) and the values of L', $K_{R(Asp)}$, and c_{Succ} listed in Table I. The best-fitting value of c_{Asp} was found to be 0.035 instead of 0.05 and a value of $K_{R(Succ)}$ was selected which best fit the data. Although this value (0.65 mM) was slightly greater than that (0.5 mM) used to account for the ligand-promoted changes in the reactivity of the sulfhydryl groups, the small discrepancy may be attributable to the differences in temperature used for the two types of experiments.[4]

Figure 5 shows additional theoretical curves (dashed) calculated with the more general equation without the assumption that $\beta \gg 1$. For these computations, the values for L, b_{CbmP}, c_{Asp}, and c_{Succ} in Table I were used along with 0.1 mM for $K_{R(CbmP)}$.

Enzyme Activity as a Function of Carbamoyl Phosphate Concentration. As shown by Bethell et al. (1968) the activity

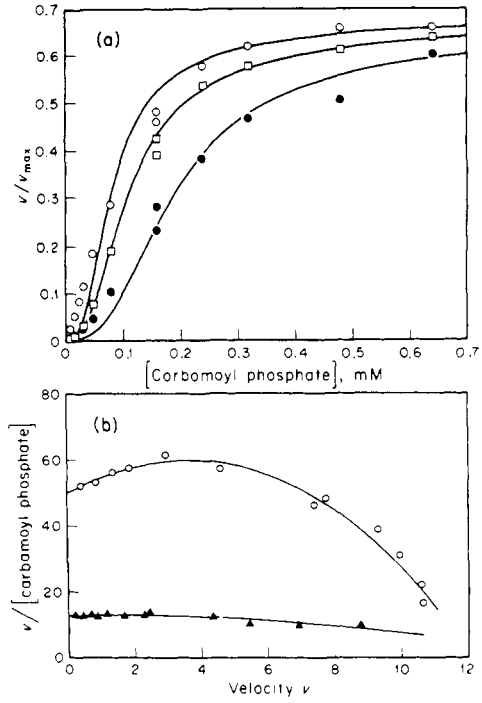

FIGURE 6: Analysis of the kinetics of ATCase (0.05 μg/mL) as a function of CbmP concentration. The measurements at 30 ± 0.2 °C were made by the procedure of Davies et al. (1970) with [^{14}C]CbmP and a fixed concentration (15 mM) of aspartate. (a) Activity measurements, v/V_{max}, in 100 mM imidazole-acetate buffer at pH 7.0 ± 0.02 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Experiments without other ligands are designated by ○; those in the presence of 2 mM ATP by □; and those with 0.5 mM CTP by ●. The curves were calculated theoretically with the parameters listed in Table I and in Results. (b) Eadie plots of kinetic data for ATCase (v/[CbmP] vs. v). The velocity, v, is expressed as μmol of carbamoyl aspartate formed h^{-1} μg^{-1} of ATCase. Experiments in imidazole buffer (described above) are designated by ○ and analogous data in 40 mM potassium phosphate buffer at pH 7.0 ± 0.02 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA are represented by ▲.

of ATCase varies in a sigmoidal fashion with CbmP concentration (in the presence of 15 mM aspartate). Experimental data confirming the results of Bethell et al. (1968) for the enzyme in imidazole-acetate buffer are presented in Figure 6a along with theoretical curves based on ordered binding of the substrates, CbmP and aspartate (Porter et al., 1969; Wedler and Gasser, 1974; Jacobson and Stark, 1975). The theoretical curves in Figure 6a were based on the parameters, b_{CbmP}, $K_{R(Asp)}$, and L, evaluated from the enzyme kinetics at varying aspartate concentration and the ligand-promoted changes in the sedimentation coefficient (Table I). Only c_{Asp} was altered (to 0.001) so as to account for the difference in buffers (see footnote 4). For V_{max} the best-fitting values were 16, 17, and 13 units/μg of enzyme in the absence of nucleotides and in the presence of ATP (2 mM) and CTP (0.5 mM), respectively, and the corresponding values for $K_{R(CbmP)}$ were 0.09, 0.18, and 0.12 mM. The large effects of ATP and CTP on $K_{R(CbmP)}$ are due presumably to direct competition of the nucleotides with CbmP at the active sites (Kleppe, 1966; Porter et al., 1969). For this reason, at low concentrations of CbmP, v/V_{max} is decreased by the addition of ATP (as well as CTP). Nonetheless the curve fitting is satisfactory qualitatively in accounting for the effect of the nucleotides in terms of a reduction of L by ATP and an increase by CTP.

Effect of Phosphate on the Allosteric Properties of ATCase. Similar kinetic parameters were obtained for the enzyme in potassium phosphate and in imidazole-acetate buffers[4] when CbmP was saturating and the concentration of aspartate was

[5] It should be noted that the effect of CbmP alone on the reactivity of the sulfhydryl groups of the R subunits in ATCase is greater than the analogous effect of that ligand on the sedimentation coefficient of the enzyme. This increase in the pseudo-first-order rate constant is greater than the value expected from the calculated ligand-promoted change in the allosteric equilibrium constant (from 250 to 7). In addition to the effect resulting from the transition from the T to R conformation, there may be a local effect of CbmP on the sulfhydryl groups of the regulatory polypeptide chains (Blackburn and Schachman, 1977).

varied (Figure 1). When, however, the CbmP concentration was low the kinetic properties of ATCase in these two buffers were markedly different. This comparison is shown in Figure 6b which reproduces the data (without nucleotides) from Figure 6a plotted as velocity/[CbmP] vs. velocity (Eadie, 1942). The curvature for the enzyme in imidazole–acetate buffer is characteristic of allosteric enzymes, whereas the almost linear behavior of ATCase in phosphate buffer indicates a much lower cooperativity. This large effect of phosphate may be attributed to the binding of aspartate to the enzyme–phosphate complex (Porter et al., 1969) even in the absence of CbmP. Through the formation of this abortive ternary complex the allosteric equilibrium is shifted toward the R state and no homotropic effect is observed at low concentrations of CbmP. Evidence in support of this interpretation was obtained from sedimentation velocity measurements. Upon the addition of aspartate (15 mM) to ATCase in phosphate buffer (40 mM) there was a 1.8% *decrease* in the sedimentation coefficient whereas with the enzyme in imidazole–acetate buffer (100 mM) the addition of aspartate (15 mM) had only a very small effect (an increase of about 0.4%).

Discussion

This test of the two-state model for ATCase is analogous to that of Changeux and Rubin (1968) but it is based on recent knowledge of the structure of the enzyme, more accurate ligand-binding data, and additional kinetic and physical properties. We do not consider here other models proposed to account for the behavior of allosteric proteins (Koshland et al., 1966; Koshland, 1970; Szabo and Karplus, 1972; Wyman, 1972; Herzfeld and Stanley, 1974). Rather it is our goal to determine whether one of the simplest models involving a minimum of experimentally evaluated parameters could account for a large number of diverse observations. Even with this model we had to introduce some assumptions because of the lack of adequate experimental evidence. We do not maintain that all the experimental results reported for ATCase can be interpreted by this model. Nonetheless the model of Monod et al. (1965) postulating a ligand-promoted concerted transition of ATCase from a constrained conformation to a relaxed state does provide an excellent basis for interpreting many of the experimental results.

The model indicates that, in the absence of ligands, most of the ATCase molecules are in the T state which is more stable than the R state by 3.3 kcal/mol (the equilibrium constant, $L = [T]/[R]$, for the enzyme in phosphate buffer is 250). When both active-site ligands, CbmP and succinate, are added the population is shifted predominantly to the R state. Molecules in the R conformation sediment 3% more slowly, react with PMB 6 to 8 times more rapidly, and have an affinity at least 20-fold higher for aspartate, succinate and the bisubstrate analogue, PALA, than do the molecules in the T-state. The addition of CbmP alone increases the stability of the R state relative to the T state by 2.1 kcal/mol (the equilibrium constant is reduced to 7 from 250). Similarly the activator, ATP, which binds to regulatory polypeptide chains, causes a relative stabilization of 0.8 kcal/mol (a reduction of the equilibrium constant from 7 to 2 in the presence of CbmP or from 250 to 70 in its absence). In contrast the addition of the inhibitor, CTP, leads to the increased stabilization of the T state by 0.9 kcal/mol relative to the R state (the equilibrium constant is raised to 35 from 7 in the presence of CbmP or to 1250 from 250 in its absence). In mixtures of either of the nucleotides and CbmP the effects on the transition were the sum of those for the ligands individually. The stabilizing influence of the ligands arises from preferential binding to one or the other conformation with relative dissociation constants (from the R state compared with the T state) of 0.55, 0.81, and 1.3 for CbmP, ATP and CTP, respectively. In these calculations it was assumed that all six of the binding sites in the ATCase molecules were saturated by the individual ligands.[6]

In the analysis of the data for the evaluation of the various parameters consideration was given to both the experimental precision and the adequacy of the approximate equations. For example, the studies of enzyme kinetics at varying aspartate concentration (Figure 1) yielded accurate data and the concentration of CbmP was sufficiently high (4 mM) to warrant using eq 6 (rather than the more general eq 4). The curve fitting of these data was relatively insensitive to values of c_{Asp} less than 0.05 and was much more discriminating in terms of L'. Hence the value of 7 for L' is reliable to about ± 5%. In contrast c_{Asp} might be as low as 0.01 rather than the value of 0.05 listed in Table I. The fit of the sedimentation data for ATCase in the presence of CbmP was very good for $L' = 7$ and $d'_{PALA} = 0.05$ (a value as high as 0.07 for d'_{PALA} was also satisfactory). The sedimentation data for solutions containing no CbmP were fit satisfactorily by assigning a value of 0.55 ± 0.05 for b_{CbmP}. This uncertainty in b_{CbmP} (which describes the relative affinities of the R and T states of ATCase for CbmP) leads to a significantly greater variation in the value of L because of the relationship $L = L'/b^n_{CbmP}$ and the value of $n = 6$. Despite this imprecision in L the calculated values of ΔG have an uncertainty of only about ±10%. Since we have used the same values of c_{Asp}, b_{CbmP}, and d'_{PALA} for the enzyme in both the absence and presence of ATP and CTP, the values of L and L' are linked; consistent quantitative values for the effects of nucleotides on ΔG and $\Delta G'$ were obtained.

The treatment of the kinetic and sedimentation data for the enzyme in the presence of CbmP involved the use of eq 6, 12, and 13 which were based on the assumption that $\beta \gg 1$. The validity of these approximate equations was tested by using the more general eq 4, 10, and 11 along with the actual concentrations of CbmP and the estimate that $K_{R(CbmP)} = 0.07$ mM in 40 mM phosphate buffer at pH 7.0 and 20 °C (0.1 mM was used for the experiments at 30 °C). The theoretical curves computed in this manner do not differ appreciably from those shown in Figures 1–3 (similar conclusions apply for the theoretical curves in Figure 4).[7]

[6] Many studies have been performed on the affinity of ATCase and of the isolated subunits for a variety of ligands such as CTP, ATP, CbmP, and succinate (Changeux et al., 1968; Hammes et al., 1970; Gray et al., 1973; Matsumoto and Hammes, 1973; Rosenbusch and Griffin, 1973; Ridge et al., 1976; Suter and Rosenbusch, 1976a,b). The pattern of binding is complex and both positive and negative cooperativity have been observed. Even with the isolated catalytic subunits different results have been obtained in various laboratories. Some of the discrepancies may be attributable to technical difficulties inherent in the measurement of binding of a ligand like CbmP which decomposes during the experiment or a ligand like succinate which is so weakly bound. In addition very recent studies (Ridge et al., 1976) have shown that differences in the preparations of the enzyme (or subunits) may be responsible for the variations in binding data. As yet no satisfactory explanation is available for the discordant results. For most of the experiments used here in testing the applicability of the two-state model the ambiguities in the binding data are not a serious concern. On the one hand, many of the tests are based on the binding of PALA for which the data seem reliable; and on the other hand, the concentrations of the other ligands in affecting the PALA-promoted changes were generally sufficiently high to attain saturation of the binding sites.

[7] With the general equations and $b_{CbmP} = 0.54$ and $L = 250$, on the one hand, or $b_{CbmP} = 0.55$ and $L = 210$, on the other, the computed curves for the kinetic and sedimentation experiments were almost identical with those calculated by the approximate treatment with $L' = 7$, $b_{CbmP} = 0.55$, and $L = 250$. These differences in the parameters evaluated from the two formulations are clearly smaller than the variations stemming from the limited precision of the experimental data.

Within the limits described above the two-state model accounted for the enzyme kinetics at varying aspartate concentration (Figure 1) and the ligand-promoted changes in the sedimentation coefficient (Figures 2 and 3). The model also accounted for the effects of CbmP and succinate (alone and together) on the reactivity of the sulfhydryl groups of the regulatory subunits in ATCase (Figure 4). Finally the shift of the population of enzyme molecules to the R conformation in the presence of CbmP and succinate accounted satisfactorily for the relative activation of the enzyme by succinate at low concentrations of aspartate (Figure 5). The two different sets of theoretical curves illustrate the comparison of the approximate treatment (based on an equation similar to eq 10) with the more general treatment (for which it was not assumed that $\beta \gg 1$). Both types of computed curves accounted for the experimental data with only minor adjustments in the parameters, $K_{R(Succ)}$ and c_{Asp}.

As seen in Figure 6a the theoretical treatment accounted qualitatively for both the sigmoidal dependence of enzyme activity on CbmP concentration and the effects of ATP and CTP on the kinetics in imidazole-acetate buffer. However, from a quantitative point of view the theoretical fit of these data (especially at low concentrations) was not as satisfactory as those obtained for the other types of measurements. The theory predicted greater cooperativity than that observed experimentally and a satisfactory fit could not be achieved even by drastic variations in the parameters, L' and b_{CbmP}. Part of the discrepancy may arise from inaccuracies in the experiments.[8] In addition complications in applying the model might be attributed to the complex binding of CbmP to different classes of sites.[6] Moreover, aspartate has been shown to bind to ATCase even in the absence of CbmP (Heyde et al., 1973; Jacobson and Stark, 1975). Until the location of these bound ligands is clarified it seems premature to modify the theory so as to improve the fit of the data. It is particularly interesting that the homotropic effect exhibited by ATCase at low concentrations of CbmP in imidazole buffer was not observed when phosphate buffer was used for the kinetic measurements (Figure 6b). This lack of cooperativity is not unexpected since, as shown by the sedimentation data, many of the enzyme molecules were converted to the R conformation as a result of the binding of aspartate to the enzyme–phosphate complex (Porter et al., 1969).

Other recent experimental observations can also be interpreted readily by the two-state model. (1) The decrease in the sedimentation coefficient of ATCase upon the addition of PALA in the presence of both CbmP and ATP was only two-thirds of the change produced by the addition of PALA in the absence of any other ligand (Howlett and Schachman, 1977). According to the model (with $L' = 2$) about one-third of the ATCase molecules are already in the R state when CbmP and ATP are present; hence the subsequent addition of PALA promotes the T to R transition for only those molecules (two-thirds) which had been in the T state. (2) When subsaturating amounts of PALA are added to the enzyme the reaction of ATCase with PMB was biphasic (Blackburn and Schachman, 1977). This result, which is in marked contrast with that observed for the enzyme in the presence of CbmP and succinate (Gerhart and Schachman, 1968), would be expected for a mixture of molecules coexisting in two conformations with sulfhydryl groups of differing reactivities. The progressive addition of PALA promoted the conversion of molecules from the T to the R conformation. (3) The affinity of ATCase for CTP is decreased upon the addition of PALA (Gray et al., 1973). Since the enzyme is almost completely converted to the R state by the addition of PALA, the decreased affinity for CTP is to be expected. (4) Hybrid ATCase-like molecules containing one native and one chemically modified, inactive catalytic subunit (along with three native regulatory subunits) exhibited allosteric properties (Gibbons et al., 1974). The lowered activity and cooperativity as well as the increased apparent K_m were interpretable in terms of the two-state model merely by changing the number of active sites from six to three (Gibbons et al., 1976). Moreover, this hybrid which could bind succinate at only the three active catalytic chains (in the one native subunit) still showed the same enhancement in reactivity of all the sulfhydryl groups (on the regulatory subunits) as that observed for the native enzyme (Blackburn and Schachman, 1977). (5) Hybrids containing the same number of active and inactive catalytic polypeptide chains but in different arrangements exhibited virtually identical homotropic and heterotropic effects (Gibbons et al., 1976). In one hybrid composed of two active and four inactive chains, the two active chains were in the same catalytic subunit and therefore in direct contact with each other; whereas in the other hybrid there was only one active chain in each subunit and "communication" had to occur through the regulatory subunits. The similarity of these "isomeric" hybrids indicates that there are no discrete "cooperative units" within the ATCase molecules (Markus et al., 1971; Diesendorf, 1975) but rather that the allosteric transition promoted by ligands is concerted.

In this analysis we have stressed the correlation between the effects of ligands on the kinetic properties of the enzyme and their effects on the quaternary structure of the oligomeric protein. Even though CbmP and ATP bind to different polypeptide chains in ATCase (Gerhart and Schachman, 1965) we interpret their effects on the quaternary structure in the same manner; i.e., they both bind preferentially to the relaxed conformation and therefore promote the transition of the protein molecules to that state. These ligands may, of course, also promote changes in the secondary and tertiary structures of the individual polypeptide chains to which they bind. Indeed ligand-promoted changes in the absorption spectra (Collins and Stark, 1969, 1971; Kirschner and Schachman, 1973a,b), in the circular dichroism (Griffin et al., 1972, 1973), in the nuclear magnetic resonance (Sykes et al., 1970; Beard and Schmidt, 1973), and in the temperature jump relaxation spectra (Hammes et al., 1971; Hammes and Wu, 1971a,b) have been detected with the catalytic subunit and with intact ATCase molecules. But these spectroscopic techniques have not revealed the extent to which local conformational changes due to ligand binding are propagated throughout the entire molecule so that unliganded polypeptide chains are affected. In this respect it is worth noting that spectrophotometry has been used with hybrid ATCase-like molecules to demonstrate that an active-site ligand which binds to only the one native catalytic subunit caused an alteration in the spectrum of the other, chemically modified catalytic subunit which did bind the ligand (Y. R. Yang and H. K. Schachman, in preparation). This "communication" (cf. Kempe and Stark, 1975), mediated via the regulatory subunits, is an indication of a gross conformational change in ATCase analogous to those presented above from sedimentation velocity and chemical reactivity studies. As shown here the two-state model of Monod et al. (1965) provides a unifying framework for analyzing such ligand-promoted changes in the quaternary structure.

[8] For these studies the enzyme concentration was extremely low (about 0.05 μg/mL) and only a small amount of [^{14}C]CbmP was converted to [^{14}C]carbamoyl aspartate. The extrapolated values of V_{max} varied from one set of experiments to another and significant corrections were required for the radioactive substrate that was not convertible to product and for the nonvolatile impurities in the [^{14}C]CbmP (Davies et al., 1970).

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