A KINETIC MODEL OF COOPERATIVITY IN ASPARTATE TRANSCARBAMYLASE

M. DEMBO* AND S. I. RUBINOW, *Biomathematics Ditision Graduate School of Medical Sciences, Cornell University, and Memorial Sloan-Kettering Cancer Center, New York 10021*

ABSTRACT A relatively simple kinetic model is proposed to account simultaneously for data on the binding of carbamyl phosphate and succinate to aspartate transcarbamylase (ATCase), and for the relaxation spectrum associated with this binding. The model also accounts for measurements of the initial velocity of the reaction of ATCase with respect to aspartate and carbamyl phosphate. The principal assumption made is that ATCase consists of three identical noninteracting cooperative dimers. Ordered binding and both sequential and concerted conformational changes in the dimers are needed to account for the properties of ATCase. The values of the parameters of this model can be determined by fitting to existing experimental evidence. Various new quantitative predictions are made that can serve as additional tests of the proposed theory.

INTRODUCTION

The enzyme aspartate transcarbamylase (ATCase) has been the subject of extensive investigation aimed at understanding the nature of regulatory proteins. ATCase catalyzes the reaction of carbamyl phosphate with aspartate to form carbamyl aspartate and inorganic phosphate, the first step unique to pyrimidine nucleotide biosynthesis. The overwhelming mass of experimental evidence about ATCase has been obtained using the enzyme produced by *Escherichia coli.* We shall review briefly the properties of this particular ATCase.

The classical initial velocity experiments of Gerhart and Pardee (1) demonstrated that the binding of aspartate to ATCase is cooperative. The authors also showed that the initial velocity of the reaction was decreased by CTP and increased by ATP, and that these nucleotides bind competitively to a special class of sites on the enzyme, distinct from the aspartate binding sites. These and subsequent studies have shown that ATCase is composed of two kinds of subunits (2), a catalytic subunit that is a trimer (3–6), denoted by C_3, and a regulatory subunit (3, 6, 7) that is a dimer, denoted by R_2. Each of the polypeptide chains of the C_3 units contains an active or catalytic site. Each can bind one molecule of carbamyl phosphate or an analogue (e.g., phosphate) and one molecule of aspartate or an analogue (e.g., succinate) (5, 6). The two polypeptide chains of the R_2 units each have a nucleotide binding site (6, 8, 9) that can

*In partial fulfillment of the Ph.D. degree at Cornell University, Graduate School of Medical Sciences, New York.

interact with either CTP or ATP. Native ATCase consists of two C_3 units and three R_2 units and hence is often denoted as C_6R_6 (3–5, 7). ATCase may be reconstituted from isolated C_3 and R_2, and such reconstituted enzyme is completely functional (2). Studies utilizing crystallographic methods (10) and electron microscopy (11) reveal that in the native enzyme the two triangular C_3 units are located above and below an equatorial belt of three R_2 units. The R_2 units connect pairs of catalytic polypeptides located on different C_3 units. Thus, C_3 units are connected to each other only via the R_2 units.

Although very active catalytically, the isolated C_3 unit does not possess the cooperative properties of the native enzyme. The binding of carbamyl phosphate and succinate to it displays hyperbolic saturation curves (5, 6, 11). Studies of the initial velocity of the reaction catalyzed by C_3 have been interpreted in terms of an "ordered bi-bi" mechanism (12–15). Carbamyl phosphate must bind before aspartate, and carbamyl aspartate must dissociate before phosphate does. It has been shown that dicarboxylic acids, such as succinate, compete with aspartate, but also form inactive or "dead-end" complexes in which subsequent binding of carbamyl phosphate is blocked (15). Several inorganic ions such as chloride and phosphate also compete with carbamyl phosphate (12, 15).

Temperature jump relaxation studies of carbamyl phosphate binding to C_3 are consistent with a simple bimolecular binding mechanism (16). In the presence of excess carbamyl phosphate, the relaxation spectrum indicates that the binding of succinate to the C_3-carbamyl phosphate complex is followed by a conformational change (16).

The complex of one catalytic trimer and three regulatory dimers (C_3R_6) (17, 18) and of two catalytic trimers and two regulatory dimers (C_6R_4) (19) have been prepared. C_3R_6 is apparently very similar to C_3; it displays no cooperative effects in binding with ligands, and is unaffected by nucleotides. C_6R_4, on the other hand, is significantly affected by nucleotides, and the initial velocity is a sigmoidal function of aspartate concentration when the concentration of carbamyl phosphate is large. Remarkably, however, both the sigmoidality and the nucleotide effects are reduced by approximately one-third compared to the native enzyme. The importance of intact R_2 units for the cooperative behavior of ATCase is further illustrated by the mutant enzyme 2-thiouracil ATCase (20). In this mutant, a discrete change in the R_2 units causes the enzyme to respond to aspartate in normal Michaelis-Menten manner, although both the V_{max} and K_m determined from the response are markedly affected by CTP and ATP. As pointed out by Chan (18) and Markus et al. (21), the properties of these modified forms of ATCase are consistent with the assumption that there is a basic unit of cooperativity in ATCase, called the "allosteric unit." This hypothetical unit consists of two catalytic sites, located on opposite C_3 units, and the R_2 unit that connects them. According to this concept, cooperative interactions between catalytic sites located on the same C_3 unit are negligible.

Several studies have shown that the binding of both CTP and ATP to native ATCase is negatively cooperative (11, 22–24). Furthermore, the binding of both these ligands is given by a second-order Adair function. Thus the data are consistent with the

assumption that the nucleotide binding sites interact in independent pairs. Temperature jump relaxation studies of nucleotide binding (25) are also easily interpreted in terms of a negatively cooperative dimeric model (25). These results support the hypothesis that the dimeric allosteric unit is the basic unit of cooperativity in ATCase.

Binding of succinate to ATCase in the presence of a high concentration of carbamyl phosphate (9) demonstrates marked positive cooperativity. As pointed out by Endrenyi et al. (26), these data are in excellent agreement with a second-order Adair function, indicating that, as with the nucleotide binding sites, the aspartate binding sites interact in independent pairs.

Studies of the binding of carbamyl phosphate (27) show that only three active sites of ATCase can bind this ligand in the absence of succinate, and that the binding curve under these conditions is hyperbolic. In the presence of succinate, it was found that all six active sites become available for carbamyl phosphate binding.

Temperature jump studies of the binding of carbamyl phosphate and succinate to ATCase have detected two relaxation processes in the native enzyme that, in terms of duration and concentration dependence, are similar to the processes seen in C_3 (28). We shall refer to the associated relaxation times of these processes as "fast." One relaxation time was observed in the absence of succinate at various levels of carbamyl phosphate concentrations. The concentration dependence of the reciprocal relaxation time for this process indicates that the binding under these conditions is a bimolecular reaction. The second fast relaxation process was observed at 10 mM succinate at various carbamyl phosphate concentration levels. In this case the concentration dependence of the reciprocal relaxation time was consistent with a mechanism involving a bimolecular reaction followed by a conformational change. Neither of the fast relaxation processes was significantly affected by the presence of nucleotides.

Studies by Hammes and Wu (28, 29) have also detected relaxation processes in the native enzyme unlike anything seen in C_3, and which are significantly affected by nucleotides. These authors concluded (30) that these relaxation times arise from distinct conformation changes that are "concerted," and important to the mechanism of regulation. Both of the relaxation times, unique to native enzyme, are considerably slower than those seen in C_3, and we shall refer to these as "slow."

Many techniques, too numerous to mention, have been employed in the attempt to detect conformational changes in ATCase associated with ligand binding, and are discussed in several extensive reviews of the literature on ATCase (31–33). These studies show that all the various ligands that can interact with ATCase bring about some kind of conformational change. The largest and most readily observable change is associated with succinate binding to ATCase in the presence of excess carbamyl phosphate (34–36). This change, as measured by reactivity of the thiol groups of ATCase, and by the ATCase sedimentation coefficient, is not seen in isolated C_3 and is markedly affected by nucleotides.

A previous attempt to analyze data on the properties of ATCase quantitatively (37) used the model of Monod et al. (38). Although this model has been very successful in accounting for nucleotide and succinate effects on certain qualitative measures of the

conformational state of ATCase, it is unable to account satisfactorily either for the negative cooperative behavior of ATCase with respect to binding of ATP, CTP, and carbamyl phosphate (39), or for the relaxation spectra of ATCase (28–30), or for data concerning the effect of nucleotides on the rate of tryptic digestion of ATCase (21), or for the properties of 2-thiouracil ATCase (20).

In the next section we introduce a theoretical model of the kinetic behavior of the catalytic sites of ATCase, and explain the empirical basis for the assumptions that underlie it. The central conception of the model is that, in the absence of nucleotides, ATCase consists of three identical noninteracting cooperative subunits, as first proposed by Endrenyi et al. (26). These subunits are structurally realized by the dimeric allosteric units of Chan (18) and Markus et al. (21). The model represents the dimer in all the various liganded and conformationally changed states necessary to represent the binding studies of carbamyl phosphate (27) and succinate (9) to ATCase, the slow and fast relaxation processes observed in conjunction with these binding studies (28), and the initial velocity studies of ATCase with respect to aspartate and carbamyl phosphate (19, 40, 41). In principle, it also accounts for studies of the effect of succinate on the initial velocity of ATCase (14, 15), although we do not apply the model to such studies here.[1] Next we derive the theoretical formulas based on the model that are needed to understand equilibrium studies of ligand binding to ATCase. Then we derive the equations governing relaxation phenomena, before applying the theory to ligand binding and relaxation data, and determining the parametric values of the theory needed to represent these data. In doing so, we have found it necessary to correct the succinate binding data of Changeux et al. (9) for the "excluded volume" effect of ATCase, which is not inconsiderable. This correction is explained in the Appendix. Lastly the model is applied to the studies of the initial velocity of ATCase with respect to aspartate at different carbamyl phosphate concentrations (19, 40, 41).

DIMERIC MODEL OF ATCase

The model we propose to represent the binding, relaxation, and initial velocity studies of ATCase is based on the following set of assumptions: (*a*) ATCase consists of three identical, noninteracting cooperative units, each of which contains two catalytic or active sites. Each active site consists of two binding sites, one for a molecule of carbamyl phosphate or an analogue thereof, and one for a molecule of aspartate, or an analogue thereof. (*b*) The binding of a ligand pair to an active site is ordered: Carbamyl phosphate must bind before binding to the aspartate site can occur. Conversely, the aspartate site must be vacated before the carbamyl phosphate site is vacated. (*c*) The binding of a carbamyl phosphate molecule to the cooperative unit at either active site inactivates the other active site. (*d*) Reactivation at the second active site cannot occur until two conditions are fulfilled. First, an aspartate or succinate molecule binds to the active site at which the carbamyl phosphate molecule is bound,

[1]Nor do we include the effects of nucleotides on the behavior of ATCase.

and second, a major conformational change of the cooperative unit takes place. These two events can occur in either order, but neither event is possible if a phosphate ion substitutes for the carbamyl phosphate molecule. (*e*) The rate of interconversion between the conformation of the cooperative unit in which aspartate or succinate binding opens up the second active site, and the conformation in which binding of aspartate or of succinate does not open up the second active site is slow compared to all other transitions among the various states of the enzyme. (*f*) After both active sites are fully occupied, the cooperative unit undergoes a fast reversible conformational change. (*g*) Succinate can bind to the free cooperative unit and forms a dead-end complex unable to bind with a single molecule of carbamyl phosphate. The formation of such a dead-end complex by aspartate is negligible.

The justification for assumption *a* lies primarily in the fact that the binding isotherms of succinate, CTP, and ATP are all consistent with a second-order Adair equation, as discussed in the Introduction. Furthermore, the studies of carbamyl phosphate binding (27) are also readily interpreted in terms of a dimeric model if one assumes that within each dimer, the binding of the first molecule of carbamyl phosphate strongly inhibits the binding of the second, and that this inhibition is released by succinate. Assumption *a* is the major theoretical conception of the model, and it results in a great simplification of the mathematical considerations, since it reduces the problem of analyzing a hexamer to the problem of analyzing a dimer.

From a structural viewpoint, we believe that the basic unit of cooperativity in ATCase consists of a set of two active sites located on opposite C_3 subunits, connected by an R_2 subunit. Such an arrangement of two catalytic sites together with their connecting R_2 subunit is the allosteric unit of Markus et al. (21). In other words, communication between catalytic sites on the same C_3 unit does not take place, whereas communication between sites on opposite C_3 units is necessary for cooperativity. This view is supported by the reports that C_3 and C_3R_6 are noncooperative (17, 18), and that in C_6R_4 the cooperativity is reduced by roughly one third (19). However, the structural arrangement of the active sites is, strictly speaking, not essential to the mathematical model we present. We believe that the changes brought about by nucleotide binding to one of the R_2 units are also restricted to a single cooperative unit.

Assumption *b* is supported by the ordered binding mechanism deduced from the initial velocity studies of C_3 (12–15), as well as by the negligible succinate binding to C_6R_6 or C_3 in the absence of carbamyl phosphate, even if phosphate ion is present (9).

Assumption *c* is based solely on the observation that only three enzymatic sites bind carbamyl phosphate when succinate is absent (27).

Assumption *d* is made to accord with the observation that succinate must be present for the occurrence of complete saturation of the enzyme with carbamyl phosphate (27), and with the many qualitative observations that binding of succinate to C_6R_6 is associated with a major conformational change only if carbamyl phosphate is present (34–36).

Assumption *e* forms the basis for the interpretation of the relaxation spectrum of ATCase. It reflects our conviction that the major conformational change required by

assumption *d* separates two slowly interconverting forms of the cooperative unit. We shall show that the slow relaxation times measured by Hammes and Wu, under conditions either of excess succinate and low carbamyl phosphate concentration (28), or of excess carbamyl phosphate and low succinate concentration (29), are interpretable as different aspects of this same process.

Assumption *f* is needed to account for the rapid relaxation process observed by Hammes and Wu when both carbamyl phosphate and succinate are present in large amounts (28).

Assumption *g* is plausible because of the considerable kinetic evidence for the existence of similar dead-end complexes in C_3 (15). This assumption is needed to account for certain aspects of the carbamyl phosphate binding data and of the relaxation spectra, as we shall see when the data are considered in detail.

We introduced here a four-dimensional vector notation to indicate the state of occupancy of the two active sites of the cooperative unit. The first two positions of the vector specify the way in which a pair of ligands are bound to one catalytic site, and the second two positions refer to the occupancy of the second catalytic site. A comma is used to separate the pairs. Within each pair, the first position refers to the carbamyl phosphate site, and the second position refers to the aspartate site.

We designate carbamyl phosphate or its concentration by A, aspartate by B, phosphate by P, carbamyl aspartate by Q, and succinate by S. Whether we mean the ligand itself or its concentration will be clear from the context. If one of the four substrate binding sites on the cooperative unit is unoccupied, then a zero is assigned to its position in the vector. For example, (00, 00) denotes the free cooperative unit or its concentration, (A0, 00) denotes the state in which the cooperative unit with carbamyl phosphate is bound to one active site, and (PS, A0) denotes the state in which a phosphate-succinate complex occupies one active site, while the other contains a carbamyl phosphate molecule.

An additional complexity is introduced by the necessity of discussing states of the cooperative unit that differ only in their conformation. The state of the unit before the slow conformational change postulated in assumption *d* is denoted by parentheses enclosing the vector, while the transformed state is denoted by square brackets. Thus, (AS, 00) and [AS, 00] denote two such interconverting states with unchanged occupancy of the active sites. In the case of the fast conformational change postulated in assumption *g*, we distinguish between the two interconverting states by means of a prime, e.g., [AS, AS] and [AS, AS]′.

A graph-theoretic representation of the model, in which the states are labeled by this notation, is shown in Fig. 1. We note that because of assumptions *b*, *c*, and *d*, there are no states such as (PS, 00), (AB, PS), (A0, A0), (A0, P0), [A0, A0], [A0, P0], [P0, 00], [P0, P0], and [00, 00]. These restrictions imply that product formation from certain states is negligible. For example, dissociation of Q from [AB, 00] would give rise to [P0, 00], which is forbidden. Hence, those central complexes from which dissociation of Q is possible are denoted as "PQ" type complexes, (PQ, 00) and

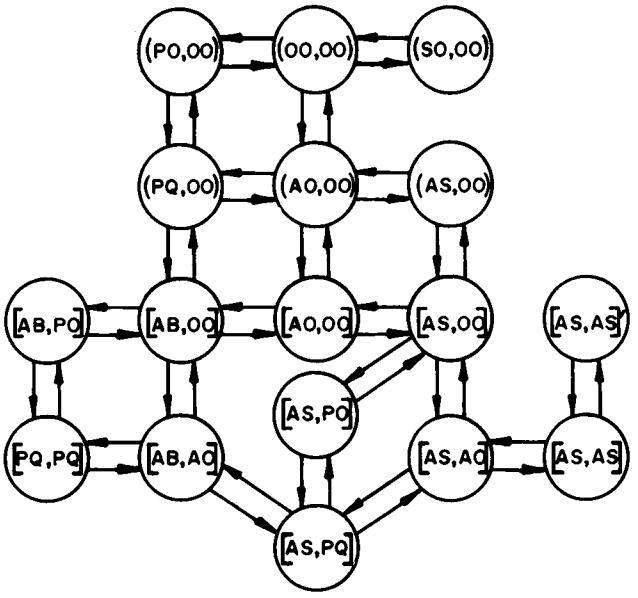

FIGURE 1 Graph of the dimeric model of ATCase, showing allowed states and transitions. The letter A represents carbamyl phosphate, B represents aspartate, S, succinate, P, phosphate, and Q, carbamyl aspartate. The vector notation for the labeling of states indicates the binding site occupancy, as explained in the text.

[PQ, PQ]. On the other hand, complexes from which dissociation of Q is blocked are denoted as "AB" type complexes, i.e., [AB, 00] and [AB, A0].

It seems plausible that the binding of aspartate to the state (A0, 00) results in the transitory state (AB, 00) followed by a change to the state (PQ, 00), from which product formation is possible. However, such changes do not alter the equations governing the initial velocity measurements, and hence, the states (AB, 00) and [AB, AB] do not have to be considered here.

The dead-end enzyme-succinate complex postulated in assumption *g* is denoted by (S0, 00) rather than (0S, 00) because Jacobson and Stark (15) suggest that this complex involves partial or complete binding of dicarboxylic acid at the carbamyl phosphate site. The analogous enzyme-aspartate complex, which would be denoted by (B0, 00), is neglected.

Fig. 1 includes two states, [AS, P0] and [AS, PQ], that arise in experiments in which ATCase interacts with carbamyl phosphate, aspartate, and succinate simultaneously. Although we have not analyzed such experiments, we include these states because we believe they provide the basis for understanding the stimulation of the initial velocity of ATCase produced by small concentrations of succinate, as follows: In the absence of succinate, and at small concentrations of aspartate, the product carbamyl aspartate is produced predominantly from the states (PQ, 00). However, in the presence of small amounts of succinate, the product can also be formed from the state [AS, PQ]. If the

affinity of aspartate for [AS, A0] is significantly greater than for (A0, 00), then it is clear that the initial velocity will become faster than in the absence of succinate. As the concentration of succinate increases, this enhancement effect disappears, because sufficient succinate is present to produce appreciable amounts of the state [AS, AS], a dead-end complex insofar as the production of product is concerned.

An interesting question left unanswered in the model is whether, in the state (P0, 00), the complementary active site is closed, as in the state (A0, 00). Without binding studies of ATCase with respect to phosphate, and for simplicity, we neglect the state (P0, P0) here. A similar ambiguity exists with regard to the production of the state (S0, S0), which is likewise neglected. We emphasize that, in either case, the states (A0, P0) and (A0, S0) are forbidden by assumptions *c* and *d*.

THEORY OF LIGAND BINDING

In the consideration of binding studies of carbamyl phosphate and succinate to ATCase in the absence of aspartate, it is necessary, as previously indicated, to consider only the subset of states of the model shown in Fig. 2. We shall call this subset of subgraph, Facet I. The figure introduces rate constants associated with the various transitions between pairs of states.

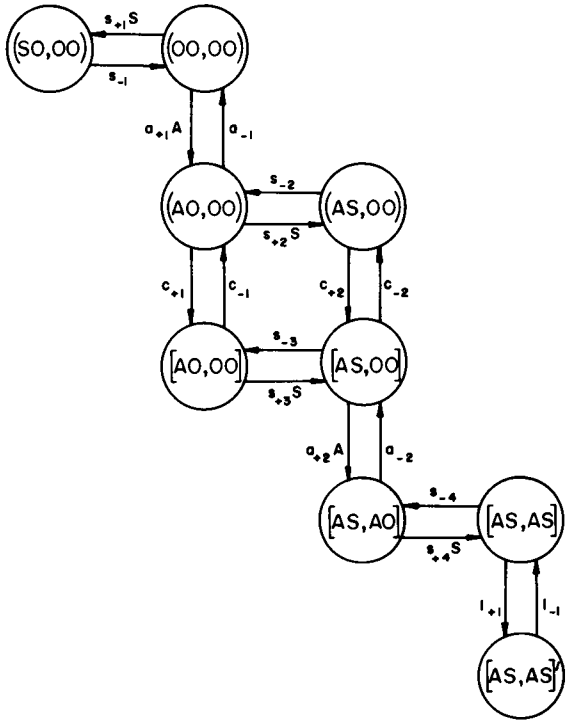

FIGURE 2 Facet I, or subgraph of model of ATCase, showing states occurring in the absence of aspartate. The labels on the directed line segments define the rate constants of the associated transitions.

Application of the law of microscopic reversibility and the conservation law for total enzyme to the graph of Fig. 2 yields the equations governing the mean number of carbamyl phosphate molecules bound per molecule of enzyme, r_a, and the mean number of succinate molecules bound per molecule of enzyme, r_s, as

$$r_a = \frac{3[K_{a1}A[1 + K_{c1} + K_{s2}S + K_{s2}K_{c2}S] + 2K_{a1}K_{s2}K_{c2}K_{a2}A^2S[1 + K_{s4}S]]}{1 + K_{s1}S + K_{a1}A[1 + K_{c1} + K_{s2}S + K_{s2}K_{c2}S + K_{s2}K_{c2}K_{a2}AS[1 + K_{s4}S]]}, \tag{1}$$

$$r_s = \frac{\begin{array}{l}3[K_{s1}S + K_{a1}K_{s2}AS[1 + K_{c2} + K_{c2}K_{a2}A] \\ \qquad + 2K_{a1}K_{s2}K_{c2}K_{a2}K_{s4}A^2S^2]\end{array}}{\begin{array}{l}1 + K_{s1}S + K_{a1}A[1 + K_{c1}] \\ \qquad + K_{a1}K_{s2}AS[1 + K_{c2} + K_{c2}K_{a2}A + K_{c2}K_{a2}K_{s4}AS]\end{array}}. \tag{2}$$

Here, the equilibrium constants are defined in terms of the rate constants of the graph as follows:

$$\begin{aligned} &K_{s1} = s_{+1}/s_{-1}, \quad K_{s2} = s_{+2}/s_{-2}, \quad K_{s3} = s_{+3}/s_{-3}, \\ &K_{s4} = (s_{+4}/s_{-4})[1 + l_{+1}/l_{-1}] \\ &K_{a1} = a_{+1}/a_{-1}, \quad K_{a2} = a_{+2}/a_{-2}, \quad K_{c1} = c_{+1}/c_{-1}, \quad \text{and} \quad K_{c2} = c_{+2}/c_{-2}. \end{aligned} \tag{3}$$

Since there is a closed loop in the model, microscopic reversibility also yields the loop relation (42)

$$K_{c1}K_{s3} = K_{c2}K_{s2}. \tag{4}$$

THE THEORY OF THE RELAXATION SPECTRUM

To understand the relaxation spectrum of ATCase, we shall first introduce some useful terminology. The states of the cooperative unit designated by the use of parentheses have a common property which we shall call condition X. Similarly, if a cooperative unit is in one of the states designated by the use of square brackets, we shall say it is in condition Y. According to assumption e, the time required either for an intra-X or intra-Y rearrangement of the cooperative unit is short compared to the time necessary for an inter-XY rearrangement. Thus the relaxation spectrum associated with the fast rearrangement of the enzyme molecules among the substates of the X conformation is represented by the following transitions:

$$(\mathrm{S0}, 00) \underset{s_{-1}}{\overset{s_{+1}S}{\rightleftharpoons}} (00, 00) \underset{a_{-1}}{\overset{a_{+1}A}{\rightleftharpoons}} (\mathrm{A0}, 00) \underset{s_{-2}}{\overset{s_{+2}S}{\rightleftharpoons}} (\mathrm{AS}, 00), \tag{5}$$

subject to the constraint that the total concentration of cooperative units for the X condition and Y condition, denoted by X_0 and Y_0, respectively, remains constant,

$$\begin{aligned} X_0 &= (\mathrm{S0}, 00) + (00, 00) + (\mathrm{A0}, 00) + (\mathrm{AS}, 00), \\ Y_0 &= [\mathrm{A0}, 00] + [\mathrm{AS}, 00] + [\mathrm{AS}, \mathrm{A0}] + [\mathrm{AS}, \mathrm{AS}] + [\mathrm{AS}, \mathrm{AS}]'. \end{aligned} \tag{6}$$

In addition, conservation of S and A during the relaxation process requires that

$$S_0 = S + (S0, 00) + (AS, 00) + [AS, 00] + [AS, A0] + 2[AS, AS] + 2[AS, AS]',$$

$$A_0 = A + (A0, 00) + (AS, 00) + [A0, 00] + [AS, 00] + 2[AS, A0] + 2[AS, AS] + 2[AS, AS]', \quad (7)$$

where S_0 and A_0 are constants.

There are three relaxation times associated with the process governed by Eq. 5, obtainable as the roots of a cubic equation. At present, it is not worthwhile to go through the exercise of obtaining these roots, because the only data available are for the special case $S = 0$. In the latter case, the reaction represented by Eq. 5 reduces to a simple bimolecular one, with relaxation time τ given by the expression

$$1/\tau = a_{+1}[A + (00, 00)] + a_{-1}. \quad (8)$$

Similarly, we see that intra-Y rearrangements of ATCase are represented as

$$[A0, 00] \underset{s_{+3}S}{\overset{s_{-3}}{\rightleftarrows}} [AS, 00] \underset{a_{+2}A}{\overset{a_{-2}}{\rightleftarrows}} [AS, A0] \underset{s_{+4}S}{\overset{s_{-4}}{\rightleftarrows}} [AS, AS] \underset{l_{+1}}{\overset{l_{-1}}{\rightleftarrows}} [AS, AS]', \quad (9)$$

subject to the constraint Eqs. 6 and 7. The scheme above yields four fast relaxation times. However, the only data available on the rearrangements of the Y state are for the special case of large S. In this case, the equilibration between [A0, 00] and [AS, 00] and between [AS, AS] and [AS, A0] is extremely rapid, so that we can assume that equilibrium is established for these pairs, i.e.,

$$[AS, 00] = K_{s3}S[A0, 00],$$
$$[AS, AS] = K_{s4}S[AS, A0]. \quad (10)$$

These equations provide two additional constraints reducing the degrees of freedom of the system, so that only two of the four relaxation times survive. If we further assume that the concentration of enzyme is small compared to the ligand concentrations, then these remaining relaxation times $\tau_\pm$ are given by

$$1/\tau_\pm = \tfrac{1}{2}[(k_{-1} + k_{+1}A + k_{+2} + k_{-2}) \pm \sqrt{(k_{-1} + k_{+1}A - k_{+2} - k_{-2})^2 + 4k_{-1}k_{+2}}], \quad (11)$$

where

$$k_{+1} = a_{+2}K_{s3}S/(1 + K_{s3}S), k_{-1} = a_{-2}[1 + (s_{+4}/s_{-4})S]^{-1},$$
$$k_{+2} = l_{+1}(s_{+4}/s_{-4})S[1 + (s_{+4}/s_{-4})S]^{-1}, \quad k_{-2} = l_{-1}. \quad (12)$$

The concentration dependence of the fast relaxation time detected by Hammes and Wu when $S = 10$ mM indicates that it corresponds to τ_- of Eq. 11. A relaxation time of

the right order of magnitude to correspond to τ_+ was also seen, but its amplitude was too small to be quantitatively studied.

As seen in Fig. 2, the graph is composed of nine states and nine transitions among them, so that the spectrum of Facet I consists of nine relaxation times. However, seven of these are fast, as discussed above (three in conjunction with Eq. 5, and four in conjunction with Eq. 9). At large times, the transients associated with these relaxation times die down, and there are consequently seven constraints of equilibrium on the subsequent behavior of the model. These, together with the constraints provided by the conservation laws for carbamyl phosphate, succinate, and enzyme, imply that the system, asymptotically for large times, has only one degree of freedom. This degree of freedom arises from the slow interconversion of the X and Y conformations. An approximate expression for the reciprocal of this slow relaxation time, obtained by neglecting terms of order E_0, is given as

$$1/\tau = (c_{+1}K_{a1}A + c_{+2}K_{a1}K_{s2}AS)/(1 + K_{s1}S + K_{a1}A + K_{a1}K_{s2}AS) + (c_{-1} + c_{-2}K_{s3}S)/(1 + K_{s3}S[1 + K_{a2}A + K_{a2}K_{s4}AS]). \quad (13)$$

This equation is derived in ref. 43, where an explicit expression for the correction term of order E_0 is also given.

COMPARISON OF FACET I WITH STUDIES OF LIGAND BINDING AND RELAXATION PROCESSES

In essence, four quantitative experiments on ligand binding to ATCase have been reported. Rosenbusch and Griffin (27) have measured the binding isotherms of carbamyl phosphate, r_a, when the succinate concentration S is 0 and 5 mM. Changeux et al. (9) have reported values of r_s, and Hammes and Wu have measured $1/\tau$ of the slow relaxation process when S = 10 mM and A is varied (28), and again when A = 1 mM and S is varied (29). These studies are represented theoretically in Eqs. 1, 2, and 13. However, before these equations were applied, it was necessary to make the following adjustments to the data.

First, the same parameter values were assumed to apply to all the experiments, although they were carried out under somewhat different conditions of buffer, pH, and

TABLE I
EXPERIMENTAL CONDITIONS OF STUDIES OF LIGAND BINDING

Study	Buffer	Temp.	pH	Authors
		°C		
Carbamyl phosphate binding	40 mM imidazole acetate	26	7.0	Rosenbusch and Griffin (27)
Succinate binding	40 mM potassium phosphate	21	7.0	Changeux et al. (9)
Relaxation spectra	100 mM potassium acetate	28	7.4	Hammes and Wu (28, 29)

temperature, as indicated in Table I. Furthermore, the half-life of carbamyl phosphate in the experiments of Changeux et al. was taken to be 6 h (slightly longer than the 5-h half-life reported by Rosenbusch and Griffin, because of the lower temperature of the experiments of Changeux et al.). The dialysis time in these experiments ranged from 12 to 18 h, and the initial concentration of carbamyl phosphate was 4 mM. Therefore, the assumption of a 6-h half-life means that the concentration of carbamyl phosphate at the time of measurement ranged between 0.5 and 1.0 mM. In addition, the dialysis data of Changeux et al. were corrected for the effect of finite volume of ATCase, as explained in the Appendix.

After the above-mentioned adjustments were made, the parameters appearing in Eqs. 1, 2, and 3 that remain to be determined are K_{s1}, K_{s2}, $(K_{c1}K_{s3})$, K_{s4}, K_{a1}, K_{a2}, c_{+1}, c_{-1} and c_{-2}. The value of c_{+2} is obtained from these by means of Eq. 4. The experiments were fitted individually to the appropriate equations by the nonlinear least squares algorithm of Bevington (44). This resulted in an overdetermined set of equations for the nine free parameters. To overcome this difficulty, parameters were successively "relaxed," and the determination was made by eye as to how much relaxation could permit a "good" fit to be retained. By iterative variation of the relaxed parameters, a single "best" set of parameter values that gave a satisfactory fit to the data was obtained. This best set of parameter values is shown in Table II. The uncertainty in the parameter values was estimated by the change needed to affect the fit markedly, if the other parameters remained fixed. The values and uncertainties of parameters not listed in Table II may be readily obtained with the aid of Eqs. 3 and 4.

Fig. 3 shows the carbamyl phosphate binding data of Rosenbusch and Griffin at $S = 5$ mM, as well as the fit of Eq. 1 to these data. Although the data at $S = 0$ are not shown, the curve for this concentration given in Fig. 3 is the best fit to five experiments, as reported by these authors. The other curves in this figure are projections representing the carbamyl phosphate binding curves expected at intermediate and large succinate concentration levels, according to Eq. 1. As we see from the curve for $S = 10$ mM, the model predicts that carbamyl phosphate binding will show positive cooperativity at very large succinate concentrations.

The ordinate intercepts of the curves in Fig. 3 are given by the limiting expression

$$\lim_{A \to 0} (r_a/A) = 3K_{A1}[1 + K_{c1} + K_{s2}(1 + K_{c2})S]/(1 + K_{s1}S). \qquad (14)$$

If there were no dead-end complex formed by the binding of succinate to ATCase, then $K_{s1} = 0$, and according to the above formula, the intercept for $S = 5$ mM would be

TABLE II

NUMERICAL VALUES OF SOME PARAMETERS OF FACET I, BASED ON BINDING STUDIES AND SLOW RELAXATION TIME STUDIES

$K_{s1} = 0.42\ (\pm 0.1)\text{mM}^{-1}$	$K_{s4} = 3.3\ (\pm 1.0)\text{mM}^{-1}$	$c_{+1} = 47.5\ (\pm 3)\text{s}^{-1}$
$K_{s2} = 0.03\ (\pm 0.03)\text{mM}^{-1}$	$K_{a1} = 72\ (\pm 15)\text{mM}^{-1}$	$c_{-1} \geqq 4{,}000\ (\pm 1{,}000)\text{s}^{-1}$
$K_{c1}K_{s3} = 0.3\ (\pm 0.03)\text{mM}^{-1}$	$K_{a2} = 1.8\ (\pm 0.3)\text{mM}^{-1}$	$c_{-2} = 4.75\ (\pm 3)\text{s}^{-1}$

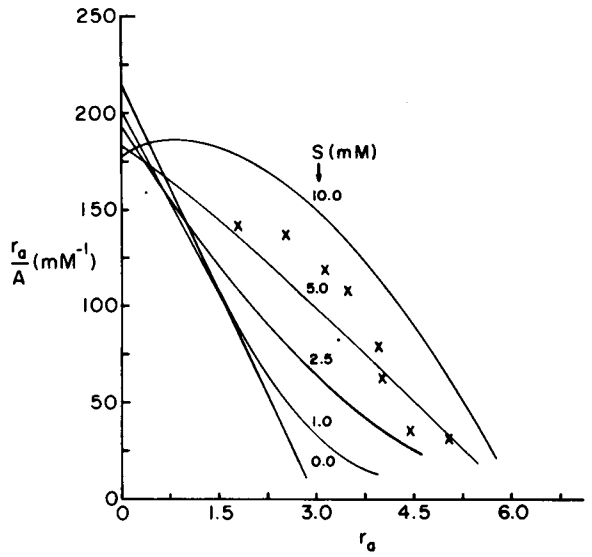

FIGURE 3 Scatchard plots representing the binding of carbamyl phosphate to ATCase, in the presence of different concentrations of succinate, S. The solid curves are based on Eq. 1 and the parameter values of Table II. The data points are those of Rosenbusch and Griffin (27) for ligand binding at S = 5 mM. Data for S = 0 are not shown, but the curve reported as representing the best fit to these data coincides with the curve labeled S = 0.

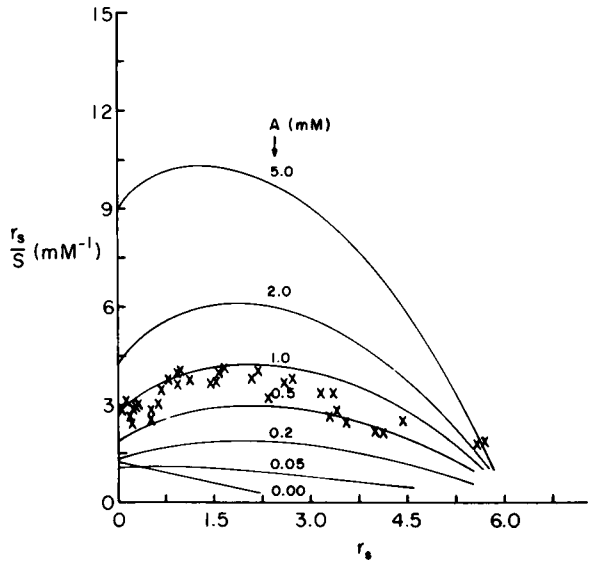

FIGURE 4 Scatchard plots representing the binding of succinate to ATCase at various carbamyl phosphate concentrations, A. The solid curves are based on Eq. 2, with the parameter values in Table II. The data points are those of Changeux et al. (9), for A between 0.5 and 1 mM, after correction for the excluded volume effect (see Appendix).

significantly greater than the intercept for $S = 0$. However, the data for $S = 0$ and $S = 5$ mM indicate that the intercept actually decreases slightly with increasing S. This failure to increase is the major reason that assumption g (existence of a dead-end enzyme succinate complex) is necessary.

Fig. 4 shows the succinate binding data of Changeux et al., together with the theoretical binding curves based on Eq. 2, from the parameter values in Table II. As explained earlier, the carbamyl phosphate concentration in this investigation was between 0.5 and 1.0 mM, and as is shown, the data points fall between the binding isotherms expected for these two concentrations. We note that the ordinate intercept of the curves increases with increasing A. Theoretically, this increase could continue indefinitely, and reflects the fact that succinate can't be released by ATCase from the state [AS, A0] to which it is driven in the presence of large concentrations of carbamyl phosphate. However, it is probable that at large carbamyl phosphate concentrations, secondary effects such as formation of the states (AA, 00) or [AS, AA], not considered in this model, become important. As the carbamyl phosphate concentration decreases, we see from the decrease in curvature of the curves in Fig. 4 that the degree of positive cooperativity gradually decreases, and that finally, as A approaches 0, the succinate binding curve actually indicates the existence of negative cooperativity.

Fig. 5 shows the measurements of Hammes and Wu (28) of the slow relaxation time of ATCase when the succinate concentration is 10 mM and the carbamyl phosphate concentration is varied. Comparison with the curve labeled $S = 10$ mM shows the

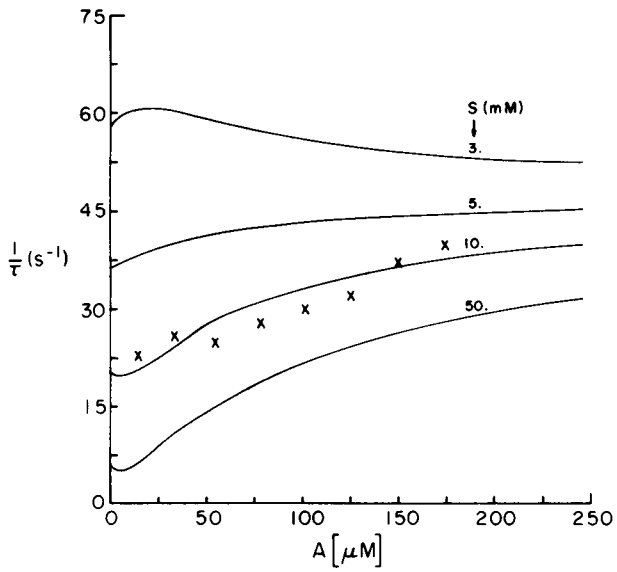

FIGURE 5 The dependence of the reciprocal of the slow relaxation time of ATCase on carbamyl phosphate concentration, at fixed values of succinate concentration S. The theoretical curves are based on Eq. 13 with the parameter values of Table II. The data points represent the experiments of Hammes and Wu (28), performed at $S = 10$ mM and the enzyme concentration $E_0 = 5(\pm 2)\,\mu$M.

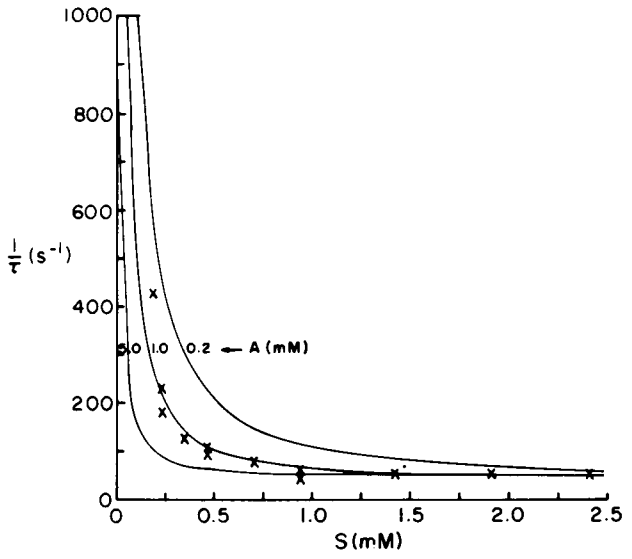

FIGURE 6 The dependence of the reciprocal of the slow relaxation time of ATCase on succinate concentration at fixed values of carbamyl phosphate concentration, A. The theoretical curves are based on Eq. 13 with the parameter values of Table II. The data points represent the experiments of Hammes and Wu (29) for A = 1.0 mM and E_0 = 16 μM.

good fit of the theory based on Eq. 13 to these data. The other curves in the figure indicate the predictions of the theory to similar experiments performed at other succinate concentrations. It should be kept in mind that Eq. 13 was derived on the assumption that the enzyme concentration is small compared to the carbamyl phosphate concentration. If this ratio is no longer small, whether because the enzyme concentration is increased or A is decreased, then the correction term to Eq. 13, given in ref. 43, becomes applicable.

In Fig. 6 the data of Hammes and Wu (29) for the reciprocal of the slow relaxation time when A = 1 mM and S is varied are shown. Also shown is the good fit of Eq. 13 to these data, as well as predictions of the theory when A is raised to 5 mM or lowered to 0.2 mM. The data in Fig. 6 show that as S approaches 0, the time required for equilibrium between the X and Y conformations becomes very short. According to the model, the reason for this is that at low S, the state [A0, 00] represents an appreciable fraction of the molecules in the Y state. The transition between the states [A0, 00] and (A0, 00) is rapid (see values of c_{+1} and c_{-1} in Table II), and τ approaches the relaxation time for this transition.

A consequence of this faster achievement of equilibrium at low S is that assumption e and in particular the assumed slow rate of interconversion which underlies Eq. 13 may begin to break down. Nonetheless, it is remarkable that no other relaxation processes were observed in these experiments, even at small succinate concentrations. Thus, on a purely empirical basis, it seems that there is only one degree of freedom in this system, so that assumption e, or some slightly modified version of it, continues to

TABLE III

NUMERICAL VALUES OF PARAMETERS OF FACET I, BASED ON FAST RELAXATION TIME STUDIES

$a_{+1} = 3 \times 10^7 M^{-1}s^{-1}$	$a_{-1} = 420\ s^{-1}$
$a_{+2} = 1.3 \times 10^7 M^{-1}s^{-1}$	$a_{-2} = 7.2 \times 10^3 s^{-1}$
$l_{+1} = 2.4 \times 10^3 s^{-1}$	$l_{-1} = 1.9 \times 10^3 s^{-1}$

$$s_{+4}/s_{-4} = 1.44\ (mM)^{-1}$$

hold. Hence, it is not necessary to invoke the various additional relaxation times that might arise in this limit until observations reveal the behavior of ATCase there.

As discussed previously, Hammes and Wu (28) have detected a fast relaxation process associated with the simple bimolecular binding of the first carbamyl phosphate molecule, with the reciprocal relaxation time given by Eq. 8. From the value of the binding constant K_{a1} that we have found for this process, which fixes the ratio of a_{+1} to a_{-1}, and the slope and intercept of the Hammes-Wu plot of $1/\tau$ versus $[A+ (00, 00)]$, we infer the estimates of a_{+1} and a_{-1} shown in Table III. It is true that a_{+1} and a_{-1} can be evaluated on the basis of the relaxation data alone, as was done by Hammes and Wu, but the uncertainties in these parameters obtained in this way are greater than if $K_{a1} = a_{+1}/a_{-1}$ is assumed to be known on the basis of the binding data, which consistency requires in any case. Similarly, the values of K_{a2}, K_{s3} and K_{s4} from Table II were utilized in fitting the Hammes-Wu data relating the fast relaxation time τ_-, associated with the negative square root in Eq. 11, to A when S = 10 mM. The estimates of $a_{+2}, a_{-2}, l_{+1}, l_{-1}$, and (s_{+4}/s_{-4}) obtained by this procedure are given in Table III. Fig. 7 displays the fit that these parameter values afford these data, and shows the predictions of Eq. 11 for the outcome of a similar experiment performed at 1-mM succinate. We note that the parameters in Table III, in conjunction with Eq. 11,

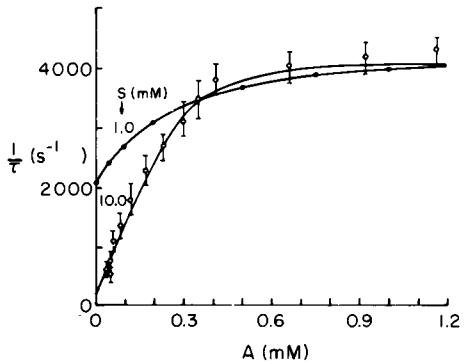

FIGURE 7 The dependence of the reciprocal of the fast relaxation time $1/\tau_-$ of ATCase on carbamyl phosphate concentration A. The theoretical curves are based on Eq. 11 with the parameter values of Table II. The data points represent the experiments of Hammes and Wu (28) for S = 10 mM and E_0 between 5 and 15 μM. In their representation of the data, these authors included a small unspecified contribution of free enzyme to the abscissa, which we have ignored here.

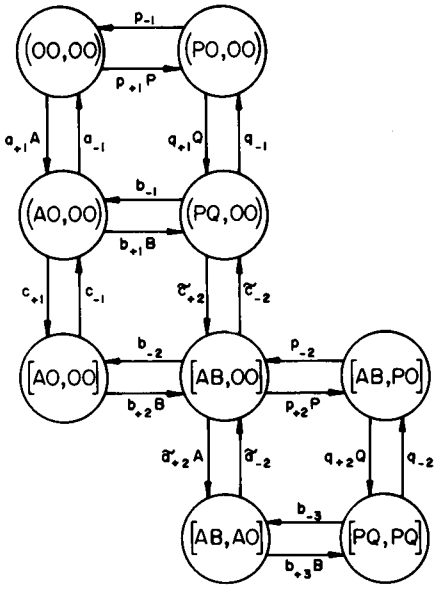

FIGURE 8 Facet II, or subgraph of model of ATCase, showing states occurring in the absence of succinate, with the labels on the directed line segments defining the rate constants of the associated transitions.

also provide the basis for quantitative prediction of the value of τ_+, although we do not explicitly present such predictions.

THEORY OF INITIAL VELOCITY OF ATCASE

In the absence of succinate, the graph of the model of Fig. 1 reduces to the subgraph shown in Fig. 8, which we call Facet II. The rate constants associated with the various transitions between pairs of states are also shown there. Because of the three states (00, 00), (A0, 00), and [A0, 00] common to both Facet I and Facet II, the theoretical representation of initial velocity experiments and the binding and relaxation studies are constrained with respect to each other. The initial velocity v of carbamyl aspartate formation in the presence of ATCase, per unit of enzyme concentration, is given according to Michaelis-Menten theory as

$$v = 3[q_{-1}W(\mathrm{PQ}, 00) + q_{-2}W[\mathrm{PQ}, \mathrm{PQ}]][\Sigma W]^{-1}, \tag{15}$$

where

$$\Sigma W \equiv W(00, 00) + W(\mathrm{A0}, 00) + W(\mathrm{PQ}, 00) + W(\mathrm{P0}, 00) + W[\mathrm{A0}, 00] + W[\mathrm{AB}, 00] + W[\mathrm{AB}, \mathrm{A0}] + W[\mathrm{PQ}, \mathrm{PQ}] + W[\mathrm{AB}, \mathrm{P0}].$$

Here, the symbols of the form $W(,)$ or $W[,]$ represent the relative weights of the various states of the system. For example, the probability that an enzyme molecule is

in the particular state [AB, 00] (after the quasi-steady state of the reaction process has been achieved) is given as

$$\text{prob}\,[\text{AB}, 00] = W[\text{AB}, 00]/\Sigma W. \tag{16}$$

The quantities $W(,)$ and $W[,]$ may be readily calculated in terms of the elementary rate constants defined in Fig. 8, by standard graph-theoretical methods (39), with the result that

$$\begin{aligned}
W(00, 00) &= [1 + J_1K_{b1}B + J_2K_{b3}B + J_1J_2K_{b1}K_{b3}B^2] \\
&\quad \times \{1 + (\tilde{c}_{-2}/b_{-2}) + [\tilde{c}_{+2}/(q_{-1} + b_{-1})] + [(q_{-1} + b_{-1})/b_{-1}]K_{b1}B\}, \\
W(\text{A}0, 00) &= K_{a1}A[1 + J_2K_{b3}B] \\
&\quad \times \{1 + (\tilde{c}_{-2}/b_{-2}) + [\tilde{c}_{+2}/(q_{-1} + b_{-1})] + (\tilde{c}_{+2}/c_{+1})[(q_{-1} + b_{-1})/b_{-1}]K_{b1}B\}, \\
W(\text{PQ}, 00) &= K_{a1}A[1 + J_2K_{b3}B] \\
&\quad \times \{1 + (\tilde{c}_{-2}/b_{-2}) + (\tilde{c}_{+2}/b_{-1}) + (\tilde{c}_{+2}/c_{+1})[(q_{-1} + b_{-1})/b_{-1}]K_{b1}B\}K_{b1}B, \\
W[\text{A}0, 00] &= K_{a1}AK_{c1}[1 + J_2K_{b3}B] \\
&\quad \times \{1 + (\tilde{c}_{-2}/b_{-2}) + [\tilde{c}_{+2}/(q_{-1} + b_{-1})] + (\tilde{c}_{+2}/c_{+1})K_{b1}B\}, \\
W[\text{AB}, 00] &= K_{a1}AK_z[1 + J_2K_{b3}B] \\
&\quad \times \{1 + \tilde{c}_{-2}b_{-1}/b_{-2}(q_{-1} + b_{-1}) + \tilde{c}_{+2}/(q_{-1} + b_{-1}) + (\tilde{c}_{+2}/c_{+1})K_{b1}B\}K_{b1}B, \\
W[\text{AB}, \text{A}0] &= K_{a1}\tilde{K}_{a2}A^2K_z \\
&\quad \times \{1 + \tilde{c}_{-2}b_{-1}/b_{-2}(q_{-1} + b_{-1}) + \tilde{c}_{+2}/(q_{-1} + b_{-1}) + (\tilde{c}_{+2}/c_{+1})K_{b1}B\}K_{b1}B, \\
W[\text{PQ}, \text{PQ}] &= K_{b3}BW[\text{AB}, \text{A}0], \\
W(\text{P}0, 00) &= (q_{-1}/p_{-1})W(PQ, 00), \\
W[\text{AB}, 00] &= (q_{-2}/p_{-2})W[\text{PQ}, \text{PQ}],
\end{aligned} \tag{17}$$

where $J_1 \equiv q_{-1}/a_{-1}$, $J_2 \equiv q_{-2}/\tilde{a}_{-2}$, $K_z \equiv (\tilde{c}_{+2}/\tilde{c}_{-2})(q_{-1} + b_{-1})/b_{-1}$, $K_{b1} \equiv b_{+1}/(b_{-1} + q_{-1})$, $K_{b3} \equiv b_{+3}/(b_{-3} + q_{-2})$, $\tilde{K}_{a2} \equiv \tilde{a}_{+2}/\tilde{a}_{-2}$. Here, b_{+2} has been eliminated by the loop relation

$$\tilde{c}_{-2}b_{-1}c_{+1}b_{+2} = \tilde{c}_{+2}b_{-2}c_{-1}b_{+1}. \tag{18}$$

The rate constants appearing in these equations are defined in Fig. 8. The constants $\tilde{a}_{-2}$, $\tilde{a}_{+2}$, $\tilde{c}_{-2}$, and $\tilde{c}_{+2}$ of Facet II are analogous to the constants a_{-2}, a_{+2}, c_{-2}, and c_{+2} of Facet I, in that the states they connect are related to each other by the interchange of aspartate and succinate molecules. It seems reasonable on this basis that there should be an "order of magnitude" agreement between the corresponding elements of these two sets of constants. However, we employ tildes to emphasize that they are not actually identical.

Eq. (15) is seen to be algebraically complex, in view of Eqs. (16) and (17). As a consequence, any attempt to determine all the parameters involved only on the basis of

TABLE IV

NUMERICAL VALUES OF PARAMETERS OF FACET II, BASED ON INITIAL VELOCITY STUDIES

$\tilde{a}_{+2}/\tilde{a}_{-2} = 1.1\ (\text{mM})^{-1}$ $\qquad (p_{-1}\,q_{-1})/(p_{-1} + q_{-1}) = 1.23 \times 10^3\,\text{s}^{-1}$

$(b_{+1}/p_{-1})(p_{-1} + q_{-1})/(b_{-1} + q_{-1}) = 0.006\ (\text{mM})^{-1}$ $\qquad (p_{-2}\,q_{-2})/(p_{-2} + q_{-2}) = 3.35 \times 10^2\,\text{s}^{-1}$

$(b_{+3}/p_{-2})(p_{-2} + q_{-2})/(b_{-3} + q_{-2}) = 0.23\ (\text{mM})^{-1}$

$$\frac{\tilde{c}_{+2}}{\tilde{c}_{-2}}\frac{(b_{-1}+q_{-1})}{(p_{-1}+q_{-1})}\frac{p_{-1}}{b_{-1}} = \frac{c_{+1}}{c_{-1}}\frac{b_{+2}}{b_{+1}}\frac{(b_{-1}+q_{-1})}{(p_{-1}+q_{-1})}\frac{p_{-1}}{b_{-2}} = 5.0$$

currently available initial velocity studies would be futile. However, by utilizing the binding studies in conjunction with the above, it is possible to make reasonable estimates of a great many of the unknown parameters, and that is how we have proceeded.

The binding studies show that K_{s2} is small, or the binding of succinate to the state (A0,00) is weak. It is reasonable to suppose that aspartate binding to this state is similarly weak, or b_{-1} is greater than or comparable with q_{-1}. Therefore, to good approximation, $b_{-1}/(q_{-1} + b_{-1}) \sim 1$, and this equivalence makes the term in curly brackets a common factor of the probability weights appearing in Eqs. (17). Hence this factor cancels itself in Eq. (15), and simplifies the fitting procedure to the initial velocity data. In fact, it is easy to see that a number of other limits lead to the same algebraic cancellation, for example, if the transition between the states (PQ,00) and [AB,00] is forbidden, so that $\tilde{c}_{+2} = \tilde{c}_{-2} = 0$. In addition, we assume $\tilde{a}_{-2} = a_{-2}$, and choose for it and a_{-1} the values given in Table III. Similarly, we fix K_{a1} and K_{c1} as given in Table II, according to the binding studies.

With the above constraints, the initial velocity v, given by Eq. 15, is expressible in terms of the six parameters listed in Table IV. Their values, also shown in Table IV,

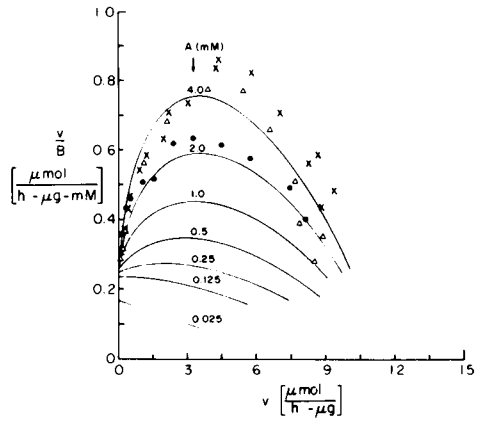

FIGURE 9 Scatchard plots representing the initial velocity of ATCase v with respect to aspartate concentration B at fixed carbamyl phosphate concentration A. The theoretical curves are based on Eq. 15 with the parameter values given in Table IV, subject to the approximations discussed under Theory of Initial Velocity of ATCase. The data points represent experiments of Gibbons et al. (40) (Δ) and Yang et al. (19) (×) at A = 4 mM, and of Kirschner and Schachman (41) (●) at A = 2 mM. All these experiments were carried out in 50 mM acetate buffer, at pH 7 and at 30°C.

were determined by simultaneously fitting all the initial velocity data shown in Fig. 9, with the least square criterion and the algorithm of Bevington (44).

The experiments described by the Scatchard plots of Fig. 9 were performed at two different concentrations of carbamyl phosphate, although all other conditions and techniques were identical. The data at 4 mM carbamyl phosphate are from Yang et al. (19) and Gibbons et al. (40). The agreement between these two studies permits greater confidence in them. The data of Kirschner and Schachman (41) were obtained at a carbamyl phosphate concentration of 2 mM.[2] These data show clearly that the curvatures of the Scatchard curves increase with increasing carbamyl phosphate concentration, while the intercepts of the curves with the ordinate axis, and most likely with the abscissa axis, do not significantly change with changing carbamyl phosphate concentration. These properties are clearly simulated by the theoretical curves, and yield strong support for the hypotheses underlying the model. For example, if product formation were permitted from the state $[AB, A0]$, then the ordinate intercept would increase with increasing A.

CONCLUSIONS

In the past, theoretical attempts to understand the kinetic behavior of ATCase have been limited to one or at most a small number of phenomena associated with the enzyme, for example, relaxation studies, binding studies, nucleotide studies, etc. Clearly, a long-range goal of such attempts is a single model that would comprehend all such studies. In view of the known complexity of structure and the number of active sites of ATCase, the attainment of this goal is beset by many obstacles. We have presented a model that we consider to be a reasonable first step in achieving this objective. We attempt only to make consistent the presently known results of binding, relaxation, and initial velocity studies of ATCase, in the absence of nucleotides. Also, we have not explicitly considered studies of ATCase in the simultaneous presence of carbamyl phosphate, aspartate, and its analogue succinate, although we have suggested how the model is extended so as to represent such studies.

The successful representation of the data by the theory, as presented herein, suggests that the underlying assumptions of the model are correct. The principal conceptions supported are first, ATCase consists of three noninteracting cooperative dimers; second, ligand binding to the enzyme is highly ordered, so that a great many bound states formally conceivable do not in fact occur; third, both sequential conformational changes, as suggested by Koshland et al. (45), and concerted conformational changes, as suggested by Monod et al. (38), are needed to account for the behavior of ATCase.

According to the theory, a single slow concerted conformational change is all that is required to understand the relaxation spectrum of ATCase. In this sense, it resembles the theory of Monod et al. However, there remain substantial conceptual differences between the models. Thus, in the present theory, there is no one-to-one correspondence between the bound states occurring in each of the two conformations;

[2]Personal communication, M. Kirschner.

the concerted conformational change occurs only after a carbamyl phosphate molecule binds; even within a conformational state of the dimeric unit, cooperativity of ligand binding is permitted.

We have presented numerous theoretical curves that serve as predictions made by the model. Future experiments should not only be able to test the essential validity of these predictions, but also provide the basis for further improvements and modifications of the model.

APPENDIX

Several authors (see for example refs. 26 and 33) have commented on the fact that the number of succinate binding sites determined by Changeux et al., on the basis of their binding data, was 3.8, rather than the six sites now known to exist. This discrepancy can be explained if their data are corrected for the fact that ATCase has a finite volume.

In the typical dialysis experiment, a known concentration of radioactive succinate, C_t, is added to a dialysis cell. After equilibration, the decays per minute on the side of the cell with ATCase, D_a, and the decays per minute on the side without ATCase, D_f, are measured. The concentration of free succinate is $C_f = (C_t D_f)/(D_f + D_a)$. At equilibrium, we assume that the concentration of free succinate is the same in both chambers of the cell. Hence, the concentration of bound succinate is

$$C_b = \frac{C_t D_a}{D_f + D_a} - \frac{(1 - V_s) C_t D_f}{D_f + D_a},$$

where V_s is the fraction of the dialysis cell from which succinate is excluded due to the presence of ATCase. Dividing both sides by the molar concentration of ATCase, E_0, we obtain the number of moles bound per mole of ATCase, r_s, as

$$r_s = \frac{C_t(D_a - D_f)}{E_0(D_f + D_a)} + \frac{V_s C_t D_f}{E_0(D_f + D_a)} = \tilde{r}_s + v_s C_f.$$

Here $\tilde{r}_s$ is the number of moles bound as reported by Changeux et al., and $v_s = V_s/E_0$ is the molar specific succinate exclusion volume of ATCase in liters per mole. We obtain the correct number of binding sites for succinate from these data by assuming a value of $v_s = 850$ ($\pm$ 150) liter/mol. This assumption is reasonable, because the volume from which water is exluded by ATCase is approximately 225 liter/mol, based on the usual estimate of 0.75 ml/g for the partial specific volume of a protein. The difference of about 600 liter/mol between the water and succinate exclusion volumes is probably because succinate is a divalent anion, and has a much greater mass than water.

NOTE ADDED IN PROOF

The general validity of the model appears to receive strong qualitative support by some recent new results of Suter and Rosenbusch (46), who show that the binding curve for carbamyl phosphate when S = 10 mM is indeed positively cooperative as predicted by the theoretical curve of Fig. 3. In addition, they have demonstrated that succinate binding when the carbamyl phosphate concentration is zero is negatively cooperative, as anticipated by the theoretical curve of Fig. 4. Other data obtained for succinate binding when A = 4 mM and for carbamyl phosphate binding at S = 0 are quantitatively inconsistent with the data of Changeux et al. (9) and Rosenbusch and Griffin (27), respectively. However, these differences, as the authors point out, may be due to artifacts associated with the novel filtration assay

utilized in their studies. Their data on carbamyl phosphate binding at $S = 0$ also indicate that it is now necessary to invoke the existence of the states (AA, 00) and/or [A0, A0] to explain the three low-affinity carbamyl phosphate binding sites seen in the absence of succinate, which were previously undetected. Furthermore, a complex similar to (AA, 00) involving binding of carbamyl phosphate at the aspartate site has been previously invoked by Porter et al. (12), to help understand initial velocity studies of the catalytic subunit. As explained under Comparison of Facet I with Studies of Ligand Binding and Relaxation Processes, the existence of these states would also affect the predictions for succinate binding at high carbamyl phosphate.

REFERENCES

1. GERHART, J. C., and A. B. PARDEE. 1962. The enzymology of control by feedback inhibition. *J. Biol. Chem.* **237**:891.
2. GERHART, J. C., and H. K. SCHACHMAN, 1965. Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase. *Biochemistry.* **4**:1054.
3. WEBER, K. 1968. New structural model of E. coli aspartate transcarbamylase and the amino-acid sequence of the regulatory polypeptide chain. *Nature (Lond.).* **218**:1116.
4. WILEY, D. C., and W. N. LIPSCOMB. 1968. Crystallographic determination of symmetry of aspartate transcarbamylase. *Nature (Lond.).* **218**:1119.
5. ROSENBUSCH, J. P., and K. WEBER. 1971. Subunit structure of aspartate transcarbamylase from *Escherichia coli. J. Biol. Chem.* **246**:1644.
6. HAMMES, G. G., R. W. PORTER, and C. W. WU. 1970. Determination of the number of regulatory and catalytic sites on aspartate transcarbamylase. *Biochemistry.* **9**:2992.
7. COHLBERG, J. A., V. P. PIGIET, and H. K. SCHACHMAN. 1972. Structure and arrangement of the regulatory subunits in aspartate transcarbamylase. *Biochemistry.* **11**:3396.
8. ROSENBUSCH, J. P., and K. WEBER. 1971. Localization of the zinc binding site of aspartate transcarbamylase in the regulatory subunit. *Proc. Natl. Acad. Sci. U.S.A.* **68**:1019.
9. CHANGEUX, J. P., J. C. GERHART, and H. K. SCHACHMAN. 1968. Allosteric interactions in aspartate transcarbamylase: I. Binding of specific ligands to the native enzyme and its isolated subunits. *Biochemistry.* **7**:531.
10. WARREN, S. G., B. F. P. EDWARDS, D. R. EVANS, D. C. WILEY, and W. N. LIPSCOMB. 1973. Aspartate transcarbamylase from *Escherichia coli.* Electron density at 5.5Å resolution. *Proc. Natl. Acad. Sci. U.S.A.* **70**:1117.
11. RICHARDS, K. E., and R. C. WILLIAMS. 1972. Electron microscopy of aspartate transcarbamylase and and its catalytic subunit. *Biochemistry.* **11**:3393.
12. PORTER, R. W., M. O. MODEBE, and G. R. STARK. 1969. Aspartate transcarbamylase: Kinetic studies of the catalytic subunit. *J. Biol. Chem.* **244**:1846.
13. COLLINS, K. D., and G. R. STARK. 1969. Aspartate transcarbamylase. Studies of the catalytic subunit by ultraviolet difference spectroscopy. *J. Biol. Chem.* **244**:1869.
14. COLLINS, K. D., and G. R. STARK. 1971. Aspartate transcarbamylase: Interaction with the transition state analogue *N*-(phosphonacetyl)-L-aspartate. *J. Biol. Chem.* **246**:6599.
15. JACOBSON, G. R., and G. R. STARK. 1975. Aspartate transcarbamylase of Escherichia coli: Mechanisms of inhibition and activation by decarboxylic acids and other anions. *J. Biol. Chem.* **250**:6852.
16. HAMMES, G. G., R. W. PORTER, and G. R. STARK. 1971. Relaxation spectra of aspartate transcarbamylase: Interaction of the catalytic subunit with carbamyl phosphate, succinate and L-malate. *Biochemistry.* **10**:1046.
17. MORT, J. S., and W. W. C. CHAN. 1975. Subunit interactions in aspartate transcarbamylase: Characterization of a complex between the catalytic and the regulatory subunits. *J. Biol. Chem.* **250**:653.
18. CHAN, W. W. C. 1975. Subunit interactions in aspartate transcarbamylase. A model for the allosteric mechanism. *J. Biol. Chem.* **250**:668.
19. YANG, Y. R., J. M. SYVANEN, G. M. NAGEL, and H. K. SCHACHMAN. 1974. Aspartate transcarbamylase molecules lacking one regulatory subunit. *Proc. Natl. Acad. Sci. U.S.A.* **71**:918.
20. KERBIRIOU, D., and G. HERVE. 1973. An aspartate transcarbamylase lacking catalytic subunit interactions. *J. Mol. Biol.* **78**:687.

21. MARKUS, G., D. K. MCCLINTOCK, and J. B. BUSSEL. 1971. Conformational changes in aspartate transcarbamylase. *J. Biol. Chem.* **246**:762.
22. GRAY, C. W., M. J. CHAMBERLIN, and D. GRAY. 1973. Introduction of aspartate transcarbamylase with regulatory nucleotides. *J. Biol. Chem.* **248**:6071.
23. MATSUMOTO, S., and G. G. HAMMES. 1973. An equilibrium binding study of the interaction of aspartate transcarbamylase with cytidine 5'-triphosphate and adenosine 5'-triphosphate. *Biochemistry.* **12**:1388.
24. ALLEWELL, N. M., J. FREDLAND, and K. NIEKAMP. 1975. Calorimetric analysis of aspartate transcarbamylase from *Escherichia coli*. Binding of cytosine 5'-triphosphate and adenosine 5'-triphosphate. *Biochemistry.* **14**:224.
25. TONDRE, C., and G. G. HAMMES. 1974. Interaction of aspartate transcarbamylase with 5-bromocytidine 5'-tri, di, and monophosphates. *Biochemistry.* **13**:3131.
26. ENDRENYI, L., M. S. CHAN, and J. T. F. WONG. 1971. Interpretation of nonhyperbolic behavior in enzyme systems. II. Quantitative characteristics of rate and binding functions. *Can. J. Biochem.* **49**: 581.
27. ROSENBUSCH, J. P., and J. H. GRIFFIN. 1973. Carbamyl phosphate binding to aspartate transcarbamylase. *J. Biol. Chem.* **248**:5063.
28. HAMMES, G. G., and C. W. WU. 1971. Relaxation spectra of aspartate transcarbamylase. Interaction of the native enzyme with carbamyl phosphate. *Biochemistry.* **10**:2150.
29. HAMMES, G. G., and C. W. WU. 1971. Relaxation spectra of aspartate transcarbamylase. Interaction of native enzyme with aspartate analogs. *Biochemistry.* **10**:1051.
30. HAMMES, G. G., and C. W. WU. 1974. Kinetics of allosteric enzymes. *Annu. Rev. Biophys. Bioeng.* **3**:1.
31. SCHACHMAN, H. K. 1974. Anatomy and physiology of a regulatory enzyme—aspartate transcarbamylase. *Harvey Lect.* **68**:67.
32. JACOBSON, G. R., and G. R. STARK. 1973. Aspartate transcarbamylases. *Enzymes.* **9**:225–308.
33. GERHART, J. C. 1970. A discussion of the regulatory properties of aspartate transcarbamylase from *Escherichia coli*. *Curr. Top. Cell Regul.* **2**:275.
34. GERHART, J. C., and H. K. SCHACHMAN. 1968. Allosteric interaction in aspartate transcarbamylase. II. Evidence for different conformational states of the protein in the presence and absence of specific ligands. *Biochemistry.* **7**:538.
35. KIRSCHNER, M. W., and H. K. SCHACHMAN. 1971. Conformational changes in proteins as measured by difference sedimentation studies. I. A technique for measuring small changes in sedimentation coefficient. *Biochemistry.* **10**:1900.
36. KIRSCHNER, M. W., and H. K. SCHACHMAN. 1971. Conformational changes in proteins as measured by difference sedimentation studies. II. Effect of stereospecific ligands on the catalytic subunit of aspartate transcarbamylase. *Biochemistry.* **10**:1919.
37. CHANGEUX, J. P., and M. M. RUBIN. 1968. Allosteric interactions in aspartate transcarbamylase. III. Interpretation of experimental data in terms of the model of Monod, Wyman and Changeux. *Biochemistry.* **7**:553.
38. MONOD, J., J. WYMAN, and J. P. CHANGEUX. 1965. On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* **12**:88.
39. RUBINOW, S. I. 1975. Introduction to Mathematical Biology. John Wiley Sons, Inc., New York.
40. GIBBONS, I., J. F. FLATGAARD, and H. K. SCHACHMAN. 1975. Quaternary constraint in hybrid of aspartate transcarbamylase containing wild type and mutant catalytic subunits. *Proc. Natl. Acad. Sci. U.S.A.* **72**:4298.
41. KIRSCHNER, M. W., and H. K. SCHACHMAN. 1973. Local and gross conformational changes in aspartate transcarbamylase. *Biochemistry.* **12**:2997.
42. HEARON, J. Z., S. A. BERNHARD, S. L. FRIESS, D. J. BOTTS, and M. F. MORALES. 1959. Enzyme Kinetics. *Enzymes.* **1**:49–142.
43. DEMBO, M. 1977. A kinetic model of cooperativity in aspartate transcarbamylase. Doctoral dissertation, Cornell University, Graduate School of Medical Sciences, New York.
44. BEVINGTON, P. R. 1969. Data Reduction and Error Analysis in the Physical Sciences. McGraw-Hill Book Company, New York.
45. KOSHLAND, D. E., Jr., G. NEMETHY, and D. FILMER. 1966. Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry.* **5**:365.
46. SUTER, P., and J. P. ROSENBUSCH. 1976. Determination of ligand binding: partial and full saturation of aspartate transcarbamylase. *J. Biol. Chem.* **251**:5986.