A Model for the Assembly of Aspartate Transcarbamoylase from Catalytic and Regulatory Subunits*

Mark A. Bothwell‡ and H. K. Schachman§

From the Department of Molecular Biology and the Virus Laboratory, Wendell M. Stanley Hall, University of California, Berkeley, California 94720

A kinetic model is proposed for the assembly of aspartate transcarbamoylase from free catalytic (C) and regulatory (R) subunits. This model involving a number of pathways divides the reactions into three classes. One group involves the rapid reversible formation of the species CR, CR2, and CR3 by the successive reaction of R subunits with the C trimers. A second class of reactions involves the essentially irreversible combination of R with C to give, respectively, the complex, C2R, and the very stable product, ATCase (C3R). The third class involves reactions between two species each containing one C subunit, i.e. C with CR, CR with CR, CR with CR2, C with CR2, and C with CR3. These reactions in the third class, except for C + CR = C2R, are irreversible. Since the reactions in the first class are much faster than subsequent ones, the concentrations of CR, C2R, and CR3 throughout most of the assembly process are determined by their equilibrium constants of formation. Measurements of the concentrations of CR2 and CR3 in experiments in which the second and third classes of reactions do not occur to any significant extent indicate that the intrinsic equilibrium constants for dissociation of R from CR, C2R, and CR3 are 1.5 × 10⁵ M⁻¹ s⁻¹, 2.5 × 10⁻⁴, and 7.5 × 10⁻⁸ s⁻¹, respectively. A single equilibrium constant does not account satisfactorily for the experimental data. The reaction of R with C2R (and presumably others of the second type) can be described by a second order rate constant of 3 × 10⁴ M⁻¹ s⁻¹ (corrected for the appropriate statistical factors). Fitting the data according to the model was accomplished by assigning a single second order rate constant of 1.3 × 10⁴ M⁻¹ s⁻¹ to the third class of reactions (multiplied by the number of equivalent relative orientations of the reactants). A preliminary investigation of the kinetics of reaction of nitrated C subunits with R subunits was conducted at much higher protein concentrations using stopped flow spectrophotometry. Possible physiological implications of the proposed assembly mechanism are considered in relationship to the role of aspartate transcarbamoylase in the regulation of pyrimidine biosynthesis in Escherichia coli.

In the preceding paper (Bothwell and Schachman, 1980) detailed tests were described of a technique for studying the assembly of aspartate transcarbamoylase from catalytic and regulatory subunits. With the method, based on the use of "I-labeled subunits, "stopping" procedures for terminating the association reactions, and electrophoretic resolution of the various species, it was possible to identify and then measure the amounts of various intermediates formed during the assembly process. The newly formed *ATCase from C and R subunits (or from C and R subunits) was so stable that virtually no exchange of subunits could be detected when *ATCase, within 5 s of its formation, was challenged with an excess of unlabeled subunits. In contrast, complexes like *CR, and *CR, are so unstable that they could only be detected, and their amounts measured, in equilibrium mixtures containing a large excess of free R subunits.

Qualitatively the results obtained under different experimental conditions were found to be consistent with an assembly scheme with the following association reactions.

\[ C + R \rightleftharpoons CR \] (1)
\[ CR + R \rightleftharpoons CR_2 \] (2)
\[ CR_2 + R \rightleftharpoons CR_3 \] (3)
\[ CR + C \rightleftharpoons C_R \] (4)
\[ C_R + R \rightarrow C_R R \] (5)
\[ C_R_2 + R \rightarrow C_R R \] (6)
\[ CR + C \rightarrow C_R \] (7)
\[ CR + CR \rightarrow C_R R \] (8)
\[ CR + C \rightarrow C_R R \] (9)
\[ CR + CR \rightarrow C_R R \] (10)

Of these 16 reactions only four are considered reversible (1 through 4) because the rupture of a single binding domain between the c and r chains leads to the dissociation of the complex. The alternative pathways in this assembly scheme (cf. Fig. 1 in preceding paper (Bothwell and Schachman, 1980)) can account for the observation that *Csubunits was the principal product when *C subunits were in excess. Similarly the scheme provides a rationale for finding *CR3 as the dominant product in experiments with *C subunits at very low concentrations and R subunits in excess. Thus far only

* This investigation was supported in part by National Institutes of Health Research Grant GM 12159 from the National Institute of General Medical Sciences, by National Institutes of Health Training Grant CA 05028 from the National Cancer Institute, and by National Science Foundation Grant PCM 072-01927. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08544.

§ To whom correspondence should be addressed.

Received for publication, October 1, 1979

1 The abbreviations used are: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; CR, CR2, CR3, C_R, C_R2, and C_R3, complexes containing C and R subunits in the amounts designated by the subscripts; *C and *R, I-labeled C and R subunits; CR, general designation for the species CR, CR2, and CR3; Cs, succinylated C subunit; NIT, nitrated C subunit; K, equilibrium constant for the association of CR1 species with R to give CR2; C, catalytic polypeptide chain; r, regulatory polypeptide chain.
one reaction rate constant, that for $C_2R_2 + R \rightarrow C_3R_3$, has been measured directly (Bothwell and Schachman, 1974, 1980), and it was shown that covalently cross-linked R dimers react at the same rate as native R subunits. In this paper the rate constants for other reactions in the pathways are evaluated in a test of a model for the assembly based on the rapid and reversible formation of $CR$, $CR_2$, and $CR_3$ followed by second order reactions leading to $C_2R_2$ and $C_2R_3$. The experimental data are also used for the estimation of intersubunit “bond” strengths in the various complexes and for a consideration of the physiological implications of the assembly mechanism and the intersubunit interactions.

**RESULTS**

Evaluation of the Equilibrium Constants for the Formation of $*CR$, $*CR_2$, and $*CR_3$ from $C$ and R Subunits—As shown previously (Bothwell and Schachman, 1980) both the formation and dissociation of complexes like $*CR_2$ and $*CR_3$ were so rapid that reaction rates could not be measured by the techniques employed in these studies. Moreover, the formation of these species at great dilution of $*C$ subunits (about 1 nM) is so rapid compared to their subsequent incorporation into $CzRz$ and $CzR$ that the $*CzR$ species attain their equilibrium concentrations before any appreciable formation of $CzR_2$ or $CzR_3$ occurs. In fact, under the experimental conditions utilized in many of the kinetic experiments it appears that the $*CR$ species re-equilibrate during the assembly process at rates which are rapid compared to those of subsequent reactions. As a consequence the concentrations of the species, $*CR$, in the experimentally observable time range are determined by their equilibrium constants and the concentrations of free $*C$ and R subunits. Accordingly an attempt was made to analyze the data for Reactions 1 to 3 in terms of equilibrium constants.

At equilibrium the concentrations of the various species containing $*C$ can be expressed as

$$
[*C] = [C]/w
$$

$$
[*CR] = 3K_1[R][*C]/w
$$

$$
[*CR_2] = 3K_1K_2[R]^2[*C]/w
$$

$$
[*CR_3] = K_1K_2K_3[R]^3[*C]/w
$$

where $w = (1 + 3K_1[R] + 3K_1K_2[R]^2 + K_1K_2K_3[R]^3)$, $[R]$ is the concentration of free R subunits, $[C]$ is the total concentration of $*C$ subunits in all forms, and $K_1$, $K_2$ and $K_3$ are the intrinsic equilibrium constants for the association of the first, second, and third R subunits to the $*C$ trimers.

Initially the experimental data were analyzed with the above equations and the assumption that all the intrinsic equilibrium constants were the same. As seen in Fig. 1a, the theoretical curve based on a single intrinsic equilibrium constant of $1.5 \times 10^{-7}$ M (for dissociation of $CR$, into $CR_+$ + R) does not provide a good fit of the experimental data. Equilibrium constants of $0.75 \times 10^{-7}$ M and $3 \times 10^{-7}$ M yielded theoretical curves which were even less satisfactory.

The inadequacy of the theoretical curves based on a single intrinsic equilibrium constant may be attributable to untested systematic errors in the experimental technique. Alternatively the lack of fit could be the consequence of the restrictive assumption in the calculations that the three equilibrium constants were identical. It is interesting to note that if all three R subunits bind to $*C$ with the same intrinsic affinity, the maximum concentration of $*CR_2$ would be about 44% of $[*C]$, regardless of the value of the equilibrium constant. Experimentally, however, it was observed in a large number of experiments (with various preparations of $*C$ and different amounts of R subunits) that the maximum value of $[*CR_2]$ was only 24 to 30% of $[*C]$. If these determinations of the maximum amounts of $*CR_2$ are valid, they can be explained only by the hypothesis that the third R subunit binds to $*CR_2$ with a larger intrinsic affinity than that for the association of the second R subunit to $*CR$. Fig. 1b shows the marked improvement in the fit of the theoretical curves to the experimental data when the intrinsic equilibrium constants for the binding of the second and third R subunits to $*C$ were adjusted independently.

A reliable estimate of the equilibrium constant for the first R subunit could not be obtained because the concentration of $*CR$ was not measurable by the technique used in this work (the method yields only the sum of $[*C]$ and $[*CR]$). However, the calculations of the dependence of $[*CR_2]$ and $[*CR_3]$ on the concentration of R subunits are much more sensitive to the values of $K_2$ and $K_3$ than to the magnitude of $K_1$. Thus for...
the theoretical curves in Fig. 1b the value of $K_1$ was assumed to be about the mean of $K_1$ and $K_2$. Equally satisfactory fits were obtained when $K_1$ was assigned the same value as $K_2$. The values of the intrinsic dissociation constants evaluated by this procedure are: $K_1 = 1.5 \times 10^{-7}$ M; $K_2 = 2.5 \times 10^{-7}$ M; and $K_3 = 7.5 \times 10^{-6}$ M.

**Analysis of the Assembly of ATCase in Terms of a Model—**

The failure to obtain kinetic data for the reactions leading to the formation of the species, $^*CR_i$, would appear to preclude a kinetic analysis of the subsequent reactions leading to $^*C_2R_2$ and $^*C_2R_3$. However, the difficulty is not as great as it first appears. As shown here, a model describing the assembly of ATCase accounts for the experimental data reasonably satisfactorily. The calculations are independent of the values assumed for the rates of formation and dissociation of $^*CR_i$ as long as the forward and backward rate constants for these processes are in the ratio determined by the equilibrium constants discussed above and the rate constants for the formation of $^*CR_i$ are as large as $10^8$ M$^{-1}$ s$^{-1}$.

In principle the rate constants for Reactions 4 through 10 must be measured in order to describe completely the kinetics of assembly of ATCase according to the scheme in Fig. 1 of the preceding paper (Bothwell and Schachman, 1974, 1980). Of these reactions only one, $C_2R_i + R \rightarrow C_2R_i$, can be studied directly and its rate measured (Bothwell and Schachman, 1974, 1980). The others cannot be studied individually because the instability of the various species, such as $^*CR_i$, $^*CR_2$, and $^*CR_3$, precludes their isolation in pure form for kinetic measurements. However, some of the reactions are sufficiently similar to warrant the hypothesis that their rate constants can be related in a simple manner. For example, Reaction 5, $C_2R_i + R \rightarrow C_2R_i$, appears to be similar to Reaction 6, $C_2R_i + R \rightarrow C_2R_i$. Therefore, it is assumed in this model that the rate constant for Reaction 5 is twice that for Reaction 6. The factor of two is introduced to allow for the fact that there are two sites on $C_2R$ to which $R$ subunits may be added while there is only one site on $C_2R_2$.

In a similar manner it seems reasonable to describe Reactions 4, 7, 8, 9, and 10 by a single fundamental second order rate constant multiplied by correction factors to account for the number of possible equivalent orientations among the reactants. Thus $C_2R_i$ and $C_2R_2$ can associate in only one way; hence the rate constant for Reaction 10 is assigned the value, $k$. Then for Reaction 7, $C_2R_i + C \rightarrow C_2R_i$, and for Reaction 9, $C_2R_i + C \rightarrow C_2R_i$, the rate constant is assumed to be $3k$ since there are three equivalent rotational orientations leading to stable products. According to this scheme, Reaction 4, $C_2R_i + C \rightarrow C_2R_i$, and Reaction 8, $C_2R_i + C \rightarrow C_2R_i$, would have rate constants of $3k$ and $2k$, respectively. The reaction between $CR$ and $C$ is considered to be reversible, and we assumed that the $C_r " bonds" in CRH are ruptured with the same rate constant as those in the species $CR_r$. This assumption is probably the least satisfactory of those that have been invoked since the rate constants for the dissociation of $CR$ could not be measured directly.

With the aforementioned assumptions the assembly process can be described by a model involving only three classes of reactions. The reactions of $^*CR_i$, $^*CR$, and $^*CR_r$ with $R$ constitute the first class. They can be described by the intrinsic equilibrium constants enumerated above. Alternatively a rate constant of $10^6$ M$^{-1}$ s$^{-1}$ (or greater) can be assumed for the association reactions, and the rate constants for the dissociation reactions can be calculated from the appropriate equilibrium constants and the assumed rate constant. In the second class are the reactions between $^*CR_i$ and $^*CR$ (where $i + j = 3$ and $i, j = 0, 1, 2, 3$); for these reactions a fundamental second order rate constant, $k$, is assigned which must be

**Summary of reactions, rate constants, and equations in model for assembly of ATCase from subunits**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate constant</th>
<th>Forward</th>
<th>Backward</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C + R \rightarrow CR$</td>
<td>$k_1$</td>
<td>$k_1$</td>
<td></td>
</tr>
<tr>
<td>$CR + R \rightarrow CR$</td>
<td>$k_2$</td>
<td>$k_2$</td>
<td></td>
</tr>
<tr>
<td>$CR + C \rightarrow CR$</td>
<td>$k_3$</td>
<td>$k_3$</td>
<td></td>
</tr>
<tr>
<td>$C_2R_i + R \rightarrow C_2R_i$</td>
<td>$k_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_2R_i + C \rightarrow C_2R_i$</td>
<td>$k_5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_2R_i + C \rightarrow C_2R_i$</td>
<td>$k_6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_2R_i + C \rightarrow C_2R_i$</td>
<td>$k_7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_2R_i + C \rightarrow C_2R_i$</td>
<td>$k_8$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_2R_i + C \rightarrow C_2R_i$</td>
<td>$k_9$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_2R_i + C \rightarrow C_2R_i$</td>
<td></td>
<td></td>
<td>$k_0$</td>
</tr>
</tbody>
</table>

*Concentrations of each species at any time are calculated from the solution of the following set of equations:

\[
\begin{align*}
\frac{d[C]}{dt} &= k_1[C][R] + k_{-1}[CR] - k_{+1}[C][R] - k_{-1}[CR][C] \\
\frac{d[CR]}{dt} &= k_1[C][R] + k_{-1}[CR] - k_{+1}[C][R] \\
\frac{d[C_2R_i]}{dt} &= k_3[C][CR] + k_{-2}[C_2R_i] \\
\frac{d[C_2R_i]}{dt} &= k_4[C][CR] - k_{-3}[C_2R_i] [C] - k_{-3}[C_2R_i][C] \\
\frac{d[C_2R_i]}{dt} &= k_5[C][CR] - k_{-4}[C_2R_i][R] + k_{-4}[C_2R_i][R] \\
\frac{d[C_2R_i]}{dt} &= k_6[C][CR] + k_{-5}[C_2R_i][R] + k_{-5}[C_2R_i][R] - k_{-5}[C_2R_i][R] \\
\frac{d[C_2R_i]}{dt} &= k_7[C][CR] + k_{-6}[C_2R_i][R] + k_{-6}[C_2R_i][R] - k_{-6}[C_2R_i][R] \\
\frac{d[C_2R_i]}{dt} &= k_8[C][CR] + k_{-7}[C_2R_i][R] + k_{-7}[C_2R_i][R] - k_{-7}[C_2R_i][R] \\
\frac{d[C_2R_i]}{dt} &= k_9[C][CR] + k_{-8}[C_2R_i][R] + k_{-8}[C_2R_i][R] - k_{-8}[C_2R_i][R] \\
\end{align*}
\]

The set of differential equations resulting from this model is shown in Table I. They were solved with a first order Runge-Cutta approximation implemented with a program in Fortran language on a PDP-11/40 computer. A value of $1.3 \times 10^6$ M$^{-1}$ s$^{-1}$ was used for the rate constant, $k$.

**Assembly of ATCase from *C and Excess R Subunits—**

The results of an assembly experiment with solutions of 5 nM $C$ and 120 nM $R$ subunits are shown in Fig. 2. In these experiments the assembly was "stopped" with unlabeled $C$ subunits rather than $C_8$ subunits. As a consequence of this "chase," $^*C_8R_i$ and $^*C_8R_2$ formed which the assembly was "stopped" were converted to $^*C_8R_3$ and $^*C_8R_3$, respectively. Therefore, the theoretical curves calculated from the model correspond to the sum of $^*C_8R_i + ^*C_8R_2$, on the one hand, and to the sum of $^*C_8R_i + ^*C_8R_2$, on the other. As seen

\[2\] The rate constant was expressed previously (Bothwell and Schachman, 1973) in terms of the molarity of $C$ and $R$ sites. However, that formalism is meaningful only if the individual sites can react independently. For the present purposes that form of expression is not useful and the reported rate constant, $1.5 \times 10^6$ M$^{-1}$ s$^{-1}$, has been replaced by the value, $6 \times 10^5$ M$^{-1}$ s$^{-1}$, the observed rate constant based on the molarity of subunits.
Assembly of ATCase from Stoichiometric Amounts of *C and R Subunits—In Fig. 2, the theoretical curves provide an excellent fit of the data for these experiments in which the major product of the assembly process was *ATCase. It should be noted that, under these experimental conditions, any *C,R2 which was formed prior to the "chase" with unlabeled C subunits would have been converted rapidly to *C2R3 because of the large excess of R subunits. Thus the theoretical curve as well as the experimental data represent the concentration of *C2R3. In contrast, the other curve represents a mixture of *CR3 and *C2R3 with the latter predominating as the assembly proceeds.

Assembly of ATCase with *C in Excess—As seen in Fig. 4, the model correctly accounts for the observed predominance of the sum of *C2R3 + *CR3 under conditions of excess *C subunits (110 nm compared to 30 nm R subunits). However, the theoretical curve shows a more rapid increase in *C2R3 and *CR3 with time than was actually measured. It should be noted that the amount of R subunits in this experiment was sufficient to convert only a small fraction of *C subunits into the complexes. As a consequence, the measurements of the radioactivity in the form of complexes were not as precise as in the other experiments described above.

Assembly of ATCase at High Concentrations of Subunits—

in Fig. 2, the theoretical curves provide an excellent fit of the data for these experiments in which the major product of the assembly process was *ATCase. It should be noted that, under these experimental conditions, any *C,R2 which was formed prior to the "chase" with unlabeled C subunits would have been converted rapidly to *C2R3 because of the large excess of R subunits. Thus the theoretical curve as well as the experimental data represent the concentration of *C2R3. In contrast, the other curve represents a mixture of *CR3 and *C2R3 with the latter predominating as the assembly proceeds.

Assembly of ATCase with *C in Excess—As seen in Fig. 4, the model correctly accounts for the observed predominance of the sum of *C2R3 + *CR3 under conditions of excess *C subunits (110 nm compared to 30 nm R subunits). However, the theoretical curve shows a more rapid increase in *C2R3 and *CR3 with time than was actually measured. It should be noted that the amount of R subunits in this experiment was sufficient to convert only a small fraction of *C subunits into the complexes. As a consequence, the measurements of the radioactivity in the form of complexes were not as precise as in the other experiments described above.

Assembly of ATCase at High Concentrations of Subunits—

in Fig. 2, the theoretical curves provide an excellent fit of the data for these experiments in which the major product of the assembly process was *ATCase. It should be noted that, under these experimental conditions, any *C,R2 which was formed prior to the "chase" with unlabeled C subunits would have been converted rapidly to *C2R3 because of the large excess of R subunits. Thus the theoretical curve as well as the experimental data represent the concentration of *C2R3. In contrast, the other curve represents a mixture of *CR3 and *C2R3 with the latter predominating as the assembly proceeds.
Several experiments were performed by the stopped flow technique in order to analyze the assembly of ATCase at much higher concentrations of subunits than were used with the radioactively labeled proteins. For these experiments, nitrated C subunits (CNIT) were used, and the change in absorbance of the nitrotyrosyl residues was measured at 430 nm as the CNIT subunits were incorporated into complexes with R subunits (Kirschner and Schachman, 1973). Fig. 5 shows the results from an experiment with CNIT and R subunits at concentrations of 1.6 × 10^{-6} M and 3.8 × 10^{-6} M, respectively. As seen by the linearity of the plot, the change in absorbance occurred with first order kinetics. The reaction studied in this way accounted for the full magnitude of the change measured in static experiments by difference spectroscopy. Moreover, no other changes in absorbance were detected in the accessible time range between 10 ms and 10 s. No change in absorbance was observed upon mixing either CNIT or CNITCNITRJ with buffer. Studies with different preparations of CNIT yielded linear first order plots with half-times of reaction of 1.1 ± 0.1 s.

DISCUSSION

Analysis of the Equilibria among *CR, and Free Subunits—Although the experimental evidence for the rapidly attained equilibria among *CR3, *CR2, *CR, *C, and excess R subunits is convincing (Bothwell and Schachman, 1980) it is difficult to fit the data in Fig. 1 satisfactorily with a unique equilibrium constant for all of the individual reactions, *CR3 + R ⇒ *CR. A substantial improvement in the agreement between theory and experiment was achieved when different equilibrium constants were used for the successive association of the R subunits to the *C trimers. However, it should be noted that, despite the improvement, the calculated curves in Fig. 1b do not account for the experimental results at low concentrations of R subunits (about 0.1 μM). Under these conditions the predominant species in the mixtures would be *C and *CR with relatively little *CR2 and *CR3. Since our technique is incapable of measuring *CR and no other method for its determination has as yet been proposed, there seems to be little justification for further attempts to evaluate K1 by curve fitting.

Is the use of three different equilibrium constants in Fig. 1b warranted? Chon (1975, 1978) assumed explicitly that the binding of each R subunit to the C trimers was governed by the same equilibrium constant. He also assumed that the enzyme activity of c chains in the C subunits was altered only if the chains were bound to R subunits. Thus in a complex like CR2 two of the c chains would have values of Kc and Vmax characteristic of CR3 and the other c chain would have the same parameters as those for C subunits. However, no evidence was cited to justify these hypotheses. If the binding of one R subunit to a C chain altered the conformation of the other c chains in that trimer, then it would be reasonable to expect nonbonded c chains to have altered enzyme activities and Kc to differ from K1 (similarly K2 could be different from both K1 and Kc). It should be noted that the c chains in CR2 which are not linked to r chains have a turnover number (Vmax) characteristic of chains in ATCase rather than of the chains in free subunits (Yang et al., 1974). Also the c "bonds" in CR2 are not as strong as those in ATCase (Subramani et al., 1977). Apparently the addition of the third R subunit to C2R causes an alteration in the cR "bonds" already formed by the two R subunits in C2R. These observations supported the view that the interaction of an r chain with a c chain in C2R (or CR3) affects the conformation of not only the c chain to which it binds but of other c chains as well. Whether this influence of cR "bonds" on the nonbonded c chains is also manifested in CR and CR3 is not yet known. Hence speculations regarding this possibility must await further experimental studies. The most compelling evidence that the three equilibrium constants are not equal and that the third R subunit binds more tightly to the C subunits than the first and second is the relatively low value for the maximum concentration of *CR in experiments at varying amounts of R subunits. Consistently the maximum value of *CR was less than 30% of [*CR] whereas 44% would be expected if K1 = K2 = K3.

Kinetics of Assembly—As seen in Figs. 2 and 3, the calculated curves for the formation of *ATCase are in good agreement with the experimental data even for widely different amounts of C and R subunits. However, the model did not predict accurately the sum of *C2R2 + *CR2 in Figs. 3 and 4. It is difficult to determine whether the discrepancies between the observed and predicted kinetics are due to shortcomings in the model or the experimental technique. In view of the complexity of the process with its multitude of association reactions and the requirement for restrictive assumptions about the various rate constants and the indirectness of the experimental technique used for the analysis, it is hardly surprising that the model did not account completely for all of the experimental data. One of the most serious experimental deficiencies is the inability to measure the concentration of *CR from the data for the equilibria among *CR and the free subunits. This particular intermediate, according to the calculations for a wide range of initial concentrations, contributes most to the subsequent synthesis of *ATCase. A particularly weak aspect of the model is the estimation of the rate of dissociation of *C2R. Under many experimental conditions *C2R would not be expected to be an important intermediate. When, however, *C subunits are in excess, as in the experiment illustrated in Fig. 4, the model predicts that a major pathway of synthesis of *C3R3 and *C2R3 proceeds via the intermediate, *CR. As a consequence the predicted rate of synthesis of *C3R3 and *C2R3 is extremely sensitive to the constants assigned for the rates of formation and dissociation of *CR. For this reason poor agreement between the theoretical curves and experimental data might be anticipated. The fact that the observed rate of formation of *C3R3 is less rapid than predicted may indicate that the rate constant for the dissociation of *C2R to produce *CR and *C is larger than the value assumed. Another difficulty which hinders a more complete understanding of the assembly process is that the rates of the early association reactions, *C + R → *CR, *CR + R → *CR2, and *CR2 + R → *CR3 are too rapid to be measured. Attempts to decrease the rates by reducing the protein concentrations were not fruitful, because the equilibria are largely in the direction of dissociation when the concentrations of *C and R subunits are lower than those used in Fig. 1. If the association rate constants for these reactions are greater than 10^6 M^{-1} s^{-1} the kinetics could not be measured unless the "stopping" solution of unlabeled C subunits could be added and mixed adequately in accurately measured times less than 5 to 10 s. Obviously the replacement of the manual technique by stopped flow methods would permit an investigation of the kinetics of formation of the *CR species.

In Chon's analysis of the assembly of ATCase, based solely on the changes of enzyme activity with time, he suggested...
that the principal pathway for assembly consisted of a rate-
determining reaction between CR₂ and CR₃ followed by a
rapid elimination of two R subunits (Chan, 1975). A subse-
quent, more extensive analysis prompted Chan to propose a
model in which a number of reactions of the type, CRᵢ + CRᵢ₊₁
→ CRᵢ₊₂, occur simultaneously with the reactions,

\[ \text{CR}_i + \text{CR}_i \to \text{CR}_{i+1} \to \text{CR}_{i+2} \]

and

\[ \text{CR}_i + \text{CR}_i \to \text{CR}_{i+1} \to \text{CR}_{i+2} + 2R, \]

having particular importance (Chan, 1978). The inclusion of
these reactions in Chan's model and their exclusion in ours
constitute the principal substantive difference in the two
models. These reactions leading to complexes containing more
than three R subunits were considered by us to be sterically
unlikely in terms of the known structure of ATCase but it
should be noted that direct experimental evidence is avail-
able favoring either including or rejecting these association
reactions. Hence we leave open the possibility that reactions
like CR₂ + CR₃ → CR₄ may occur under certain circum-
stances. When the excess of R over C subunits is very great as
in Chan's studies, the initial assembly steps yield CR₁ and
CR₁ almost exclusively. Thus, under these conditions even a
sterically unfavorable reaction between these species might
assume a significant role. For the assembly in vivo, where
there is more likely to be a closer balance of C and R subunits,
the pathways we have considered are probably of greater
importance. For those conditions (Fig. 3), our model accounts
for the rate of formation of ATCase without invoking reac-
tions between *CR₃ and *CR₁.

Weaknesses in the Models—There is an assumption im-
plied in our model which is arbitrary and illustrates a lack of
understanding of fundamental aspects of the association pro-
cess. It is assumed that the reaction of C with CR, CR₂, for
example, proceeds at the same rate as the reaction between
CR₁ and CR₂. On the one hand, if there is a rigidly defined
angular orientation of a c:r “bond” in a CR complex, then it
would be impossible to align properly one R of *CR₁ with the
reacting C subunit without simultaneously aligning the two
other R's in *CR₂. According to this restriction the angular
specificity of the C + CR₁ and C + CR₂ reactions would not
differ, and the reaction rates would be the same. On the
other hand, if a “bond” is flexible, then one R of *CR₁
might react initially with C without requiring the simulta-
neous correct orientation of the other R's. In this case the
reaction between C and *CR₁ should be three times greater
than that between C and CR₁ because there are three times
the number of independently associating R's. Obviously the
data for ATCase do not permit one to distinguish
between these two alternative possibilities. If individual c:r
bonds form independently, the model of assembly would have
to be altered accordingly. Similarly alterations would be re-
quired if association reactions involving e chains (as well as R
subunits) occurred to a significant extent.

Chan's model (1976) required the fitting of a single experi-
mental curve with a theory containing four independent ad-
justable parameters. The difficulty in obtaining a unique and
correct assignment of so many independent variables from a
single empirical relationship is well known. Moreover, his
analysis depends critically on the precise knowledge of the
equilibrium constants for the reactions, CR₋₁ + R → CR₀.
Chan assumed that the three intrinsic equilibrium constants
for the binding of R's to C subunits were the same. If, as has
been suggested above, these constants are not equal, the
analysis of Chan would require major revision. It should be
noted that Chan concluded that pathways leading to the
formation of CR₃ are relatively unimportant. While this con-
clusion may be appropriate for experiments with an extremely
large excess of R subunits (as generally used by Chan), our
results and model show that CR₃ is a major kinetic interme-
diate at stoichiometric R to C ratios and a principal final
product when C subunits were in excess.¹

Interactions between C and R Subunits in ATCase—Al-
though free energies of interaction between C and R subunits
can be calculated from the equilibrium constants for the
various reactions, CR₁ + R ⇋ CR₂ these values (and espe-
cially the changes in them caused by ligands) are of limited
relevance to an understanding of the allosteric properties of
ATCase. The CR complexes exhibit neither the homotropic
nor the heterotropic effects characteristic of the native enzyme
(Mort and Chan, 1975). In contrast, hybrid molecules contain-
ing one active and one inactive C subunit along with three
native R subunits exhibit both of these allosteric effects (Gib-
bons et al., 1974). Apparently the constraint imposed on CR₃
when it is “filled in” with an inactive C subunit has a marked
effect on the intersubunit interactions (as well as the interac-
tions among the e chains in the active subunit). Even CR₃
which does exhibit both cooperativity and inhibition by CTP
(Yang et al., 1974) has c:r bonds which are weaker in strength from
those in ATCase (Subramani et al., 1977).

Since as yet it has not been possible to measure directly the
equilibrium constant for C and R subunit interactions in
ATCase, we have used the kinetic data obtained in these
assembly studies for tentative estimates. From the exchange
studies with *ATCase we estimate that the rate constants for
the dissociation of either C or R subunits from ATCase are
less than 2 × 10⁻⁸ s⁻¹. If this value is combined with the rate
constant estimated for the reaction of CR₂ + C (4 × 10⁻⁷ M⁻¹
s⁻¹) we calculate that the equilibrium constant for dissociation
of one C subunit from ATCase (CR₂ ⇔ CR₁ + C) is less than
5 × 10⁻¹⁴ M. Similarly the value for the dissociation of one R
subunit from ATCase (CR₁ ⇔ CR₀ + R) is less than 3 × 10⁻¹⁸ M.

Stopped Flow Experiments—Based on the assembly exper-
iments at very low protein concentrations (20 nM in C sub-
units) we estimate the half-time for the formation of c:r “bonds”
at the concentrations used in the stopped flow experi-
ments (20 μM in C subunits) to be about 0.02 s. The measured
half-time for the observed change in absorbance is about 1.1
s. Thus the half-time obtained from the stopped flow experi-
ments cannot be attributed directly to the rate of formation
of c:r “bonds.” What then is the process measured in the
stopped flow apparatus? The time required for the formation
of CNITCR₂ and CNITCR₃ from the rapidly formed CNITCR₁
and CNITCR₂ is estimated to be about 0.5 s. Hence it is possible
that some disproportionation reactions are responsible for
the observed reaction times. However the kinetics of these
processes are not likely to be even approximately first order,
and the observed change in absorbance follows first order kinetics
for more than 95% of the reaction. This finding could be
accounted for by a slow conformational change in the CNIT
subunits subsequent to their rapid association with R sub-
units, Wu and Hammes (1973) have proposed that an allo-
sterically important conformational change occurs in ATCase
almost this slowly. Certainly the striking changes in the kinetic

¹ It is worth noting that a small amount of CR₃ is found in virtually
all preparations of purified ATCase. Moreover, CR₃ is apparently
formed during the in vivo synthesis of ATCase. When stains for
denatured enzyme activity were used to analyze gel electrophoresis experiments
with crude extracts of derepressed E. coli, an active component with the
mobility of CR₃ was detected along with the major species corresponding to ATCase (Y. R. Yang, M. A. Bothwell, and H. K.
Schachman, unpublished observations).
properties of the C subunits resulting from their assembly in ATCase indicate that these subunits undergo a marked alteration in conformation in the process. Whether these changes are responsible for the observed rate in the stopped flow experiments is not known at present. A more detailed investigation of this possibility is warranted.

Physiological Aspects of ATCase Assembly—Escherichia coli growing logarithmically on glucose has a doubling time of about 40 min and contains about 150 ATCase molecules per cell. Hence the rate of formation of ATCase under these conditions corresponds to the production of a solution of $10^{-5}$ μM ATCase in 40 min. At this concentration, C and R subunits associate to form ATCase in vitro in less than 1 s. Thus if ATCase is formed in vivo from C and R subunits, the measured rate in vitro is sufficient to ensure that the synthesized subunits associate to ATCase. This is necessary if the accumulation of unassembled (and therefore unregulated) C subunits is to be prevented.5

Since the allosteric properties of ATCase are functionally important as a means of regulation of pyrimidine biosynthesis in E. coli (Gerhart and Pardee, 1962) and since C and CR₃ possess the catalytic capability but none of the regulatory properties of ATCase, it may be necessary to the bacteria that the fraction of catalytic chains in the form of free C subunits and CR₃ be maintained at a low level. Achieving this goal depends on both the amount of R subunits produced relative to C and also the strength of the cr “bonds.” The potential for formation of CR₃ as an active and unregulated abortive complex of assembly would appear to limit the amount of R which should be produced. At a concentration of $10^{-5}$ μM, we estimate from the model that 90% of the C subunits would form ATCase (or CR₂) within 10 s if the ratio of c to r chains is 1.0. If there is a 20% excess of r chains, this time would be increased to 50 s; and 1000 s would be required for assembly of ATCase if there was a 2-fold excess of r relative to c chains. Similarly the strength of the cr bond is seen to be of critical importance because stronger cr bonds would cause a decrease in the rate at which CR₅ (and CR₃) could be converted to ATCase. If, for example, the equilibrium constant for the association of C + R subunits were 10-fold larger, the time required to achieve 90% incorporation of C subunits (at $10^{-5}$ μM) into ATCase with a 2-fold excess of R subunits would be increased (according to the model) from $10^7$ s to nearly $10^9$ s. Thus the kinetic problems in the assembly of ATCase may have had an impact on the evolutionary processes leading to the present values of the strength of the cr “bond” and may have led to coordination of the synthesis of the catalytic and regulatory chains.

REFERENCES
Wu, C.-W., and Hammes, G. G. (1973) Biochemistry 12, 1400-1408
A model for the assembly of aspartate transcarbamoylase from catalytic and regulatory subunits.

M A Bothwell and H K Schachman