Pathways of Assembly of Aspartate Transcarbamoylase from Catalytic and Regulatory Subunits

(protein interactions/bonding domains/allosteric enzymes/kinetics)

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ABSTRACT A scheme for the assembly of aspartate transcarbamoylase (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) from catalytic and regulatory subunits is presented along with a technique for detecting intermediates and measuring the kinetics of assembly at protein concentrations of about 10 nM. ^{125}I -labeled subunits (10^6 cpm/ μg) were used, and the reaction was "stopped" at specific times with an appropriate "chase" followed by electrophoretic separation and measurement of the amounts of the various species. Two intermediates were identified. A stable enzyme complex lacking one regulatory subunit is the principal product when catalytic subunits are in excess. A transiently stable complex lacking one catalytic subunit is the principal species when regulatory subunits are in excess. Measurements with mixtures of the purified regulatory-deficient molecules and free regulatory subunits gave a second-order rate constant of $10^5 \text{ M}^{-1} \text{ sec}^{-1}$ for the formation of bonding domains between catalytic and regulatory chains. No data for the rate of rupture of these bonds are available, but the kinetics of the assembly of the enzyme *in vitro* can be accounted for if this value is about 10^{-2} sec^{-1} . Assembly from subunits occurs in seconds at concentrations equivalent to those that would exist *in vivo* even if there were only one enzyme molecule per cell.

Although considerable research has been done on the assembly of large complexes like viruses, ribosomes, flagella and membranes, there have been only a few reports about the sequence of steps in the assembly of the simpler oligomeric enzymes. The allosteric enzyme, aspartate transcarbamoylase; (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) (ATCase) from *Escherichia coli*, is ideal for such studies because it is a well-defined oligomer (1) composed of six catalytic (c) and six regulatory (r) polypeptide chains (2-6) and it can be dissociated into catalytic (C) and regulatory (R) subunits and then reconstituted in high yield (1, 7). The c chains are organized as trimers (6) and the r chains as dimers (6, 8, 9), both in the intact enzyme and after its dissociation with certain mercurials. Recent evidence from electron microscopy (10) and x-ray diffraction studies (11) has provided support for a model of ATCase (6) as a complex of two C subunits bridged by three R subunits, i.e., C_2R_3 . Ac-

cording to this model there are six c:c bonding domains linking the c chains within each of the two trimers, three r:r bonding domains linking the r chains in the three R dimers, and six c:r domains linking the c and r chains (6, 12).

At protein concentrations of several mg/ml the association of C and R, as measured by spectral changes in stopped flow experiments, is completed within seconds (13). In order to study individual steps in the assembly process and to identify and characterize possible intermediates, we turned to a sensitive technique that permitted kinetic measurements on solutions so dilute ($\mu\text{g}/\text{ml}$) that the assembly required about 30 min†. The method involved mixing, for example, ^{125}I -labeled C (10^6 cpm/ μg) with excess R followed by "stopping" the reactions at various times by the addition of unlabeled C. The various species, ^{125}I -labeled C, ^{125}I -labeled intermediates, and ^{125}I -labeled ATCase, were then resolved by electrophoresis on polyacrylamide gels and their amounts were determined (13). As shown below, this technique permits a preliminary kinetic analysis of the assembly of ATCase from its subunits and the identification of some transiently stable intermediates.

Scheme for the Assembly of ATCase. Many reactions might be implicated in the assembly of ATCase from its composite subunits; the various pathways are shown schematically in Fig. 1. ATCase can be formed by (a) the reaction of CR_3 with C, (b) the combination between CR_2 and CR, and (c) the reaction between C_2R_2 and R. Of the possible intermediates, only C_2R_2 , an ATCase-like molecule lacking one R (14, 15), is sufficiently stable to be prepared in pure form. Some of the reactions in Fig. 1 are probably reversible, but those leading to the rupture of c:r bonding domains are not considered at present. If such dissociation reactions are slow and R is in large excess during the assembly, the principal product would be CR_3 , an intermediate postulated by Chan and Mort (16) on the basis of the unusual enzymic behavior of mixtures containing C and a large excess of R.

In this scheme we have not considered other possible reactions. First, those involving individual c chains may be neglected since intact C subunits are incorporated directly into ATCase (6). Furthermore, the lack of hybridization between native and succinylated C (C_s) over extended periods of time in neutral buffers indicates that the dissociation of subunits into single chains is extremely slow. Second, pathways involving a transient association of C subunits followed by the stepwise addition of three R subunits cannot be kinetically

Abbreviations: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; c, catalytic polypeptide chain; r, regulatory polypeptide chain; CR_3 , C_2R_2 , and C_2R_3 , complexes containing C and R subunits in the amounts designated by the subscripts; *C, ^{125}I -labeled C; C_s , succinylated C; $\text{CC}_\text{s}\text{R}_3$, an ATCase-like molecule containing one C, one C_s , and three R; TNP, trinitrophenyl.

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‡ This technique, the experimental results, and a model for the assembly were presented at the 1974 ICN-UCLA Winter Conference on Assembly Mechanisms, March 3-8, 1974.

FIG. 1. Scheme for the assembly of ATCase from subunits. Only association reactions are illustrated in the various pathways even though some of the reactions are probably reversible; e.g., $CR_2 \rightarrow CR + R$.

significant. No measurable association of C is observed in sedimentation experiments even at protein concentrations of 0.1 mM. Hence, at the low concentrations (10 nM) used in our experiments the fraction existing as C_2 is too small to be kinetically important even if the subsequent association with R were to occur with a rate constant of $10^9 \text{ M}^{-1}\text{sec}^{-1}$, an upper limit for a diffusion-controlled association of protein molecules. Third, steric factors probably prevent the association of species having a total of more than three R subunits. Thus, reactions such as $CR_2 + CR_2 \rightarrow C_2R_4$ are excluded. Fourth, those involving single r chains are not considered since the combining unit in hybridization experiments is known to be the R dimer (8, 9). Since the dissociation constant of R to chains is estimated to be in the range 100–0.1 nM (6), there will be a significant fraction as single chains at the protein concentrations (10 nM) used in our experiments. Nonetheless, we assume for the present that R subunits (rather than r chains) combine directly with C. The validity of this assumption must be tested in the future with crosslinked R dimers, which combine with C to give ATCase-like complexes in high yield (6), in order to determine whether additional reactions must be added to those depicted in Fig. 1.

MATERIALS AND METHODS

Materials. ATCase and C and R subunits were prepared as described (6, 7, 12, 17). C_8 containing six succinyl residues per chain was prepared by treating C with 1.6 equivalents of succinic anhydride per lysyl residue (4, 9). Reaction of C with trinitrobenzene sulfonic acid (18) gave a trinitrophenylated protein, C_{TNP} , which, on the basis of its absorbance at 344 nm, contained two TNP-groups per chain and combined readily with R to give ATCase-like molecules. C_{TNP} was formed by adding 0.6 ml of C at 3 mg/ml in 20 mM triethanolamine-HCl buffer (pH 8.5) to 1.4 ml of a solution of trinitrobenzene sulfonic acid, at 1 mg/ml, in the same buffer; after 150 min the reaction was terminated with 2 μ l of 2-mercaptoethanol.

^{125}I -labeled C (*C), $10^6 \text{ cpm}/\mu\text{g}$, was prepared by the method of Syvanen *et al.* (19), and ^{125}I -labeled R (*R) was prepared by reacting R with ^{125}I -labeled methylparahydroxybenzimidate-HCl by the procedure of F. T. Wood and J. C. Gerhart (private communication) who kindly donated the methylparahydroxybenzimidate-HCl.

C_2R_2 (14, 15) was prepared by partial dissociation of ATCase with neohydrin (17). About 60 μ l of a neohydrin solution, 10 mg/ml in 0.1 M Tris-HCl at pH 9.25, were added to 26 mg of ATCase at a concentration of 20 mg/ml in 10 mM

FIG. 2. Kinetics of association of *C and R to form ATCase and C_2R_2 . The solutions of *C and R in 40 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 0.2 mM Zn^{2+} were mixed at 22° , and at various times, indicated on the *abscissa*, aliquots were removed and the assembly was "stopped" by the addition of an equal volume of 40 μM unlabeled C. The species were separated and counted for ^{125}I as described in *Materials and Methods*, and the results are expressed on the ordinate as % of total radioactivity converted into ATCase and C_2R_2 . In (A) the initial concentrations were 5 nM for *C and 120 nM for R. The corresponding concentrations in (B) were 100 nM and 30 nM.

Tris-HCl at pH 8.4. After 1 hr the reaction mixture, containing about 20% ATCase, 40% C_2R_2 , and 40% C, was fractionated chromatographically to give C_2R_2 , which appeared identical to that produced by reconstitution from R and C, with the latter in excess (15). Dissociation of ATCase with *p*-hydroxymercuribenzoate also produces some C_2R_2 (20).

Methods. Resolution of C and ATCase or R and ATCase was performed by polyacrylamide gel electrophoresis with 5% and 7.5% gels, respectively, with the buffer system of Jovin *et al.* (21). Hybrid ATCase-like molecules containing C and C_8 were separated with 5% gels and the discontinuous Tris-barbital buffer system described by Gabriel (22). Gels were stained with Coomassie Brilliant Blue G-250 (Serva) by the method of Diezel *et al.* (23). Sedimentation experiments were conducted with a Beckman model E ultracentrifuge equipped with a photoelectric scanner (24). Sucrose gradient sedimentation experiments were performed with a Spinco model L centrifuge and a SW 50 rotor.

Kinetic analyses of the incorporation of *C and/or *R into ATCase and intermediates were based on the assay of R

described by Syvanen *et al.* (19). This technique involved mixing, for example, *C with a large excess of R followed by the removal of aliquots at specific times and the addition of a large excess of unlabeled C . By means of this "chase," the unused R was consumed and the reaction was effectively stopped. The various species were then resolved by electrophoresis, and the amounts of *C , *ATCase , and ^{125}I -labeled intermediates were measured with a Nuclear-Chicago γ counter. The electrophoretic technique yielded a clear separation of C_2R_2 from $ATCase$; thus, the kinetics of formation of the intermediate, *C_2R_2 , in the assembly process could be measured directly. Aggregates in small amounts were detected in most experiments.

RESULTS

Conversion of *C into Intermediates and $ATCase$. Fig. 2A shows the results of a kinetic experiment with a mixture containing initially *C at 5 nM and R at 120 nM. About 80% of the radioactivity was recovered as $ATCase$ and C_2R_2 , with an additional 5% appearing as aggregates. The half-time of formation of $ATCase$ was about 5 min. In contrast, C_2R_2 was formed more rapidly, attained a maximum within 1 min, and then decreased in amount slowly during the remaining 30 min. Over the range of concentrations used in the kinetic experiments the counts in *C and *ATCase were proportional to the amounts of these components applied to the gels.

C_2R_2 as an Intermediate in the Assembly Process. As seen in Fig. 2A, the "incomplete" molecule, C_2R_2 , was detected in reaction mixtures containing a large excess of R relative to C . Fig. 2B shows that the dominant product was C_2R_2 when the reaction mixture contained an excess of C (100 nM) relative to R (30 nM). The half-time for formation of both C_2R_2 and $ATCase$ was about 5 min.

The technique used in these experiments has a serious pitfall in that the hypothetical intermediates, *CR_2 and *CR_3 , would have been converted, respectively, to *CCR_2 and *CCR_3 upon the addition of C during the "stopping" reaction. Therefore, the species identified as C_2R_2 represents the sum of C_2R_2 and CR_2 ; similarly, the product identified as C_2R_3 ($ATCase$) corresponds to the sum of C_2R_3 and CR_3 . As shown below, this ambiguity is eliminated in part by "stopping" the assembly with C_8 , which produces hybrid species that migrate to different positions from those for molecules completed before the addition of the "chase." In this way *C_2R_3 is separated from *CC_8R_3 , and *C_2R_2 is distinguished from *CC_8R_2 .

CR_3 as an Intermediate in the Assembly Process. Fig. 3 shows results obtained with C_8 as a "stopping" reagent. When the initial concentrations were 10 nM for C and 120 nM for R , the principal product was $ATCase$ with a half-time of formation about 6 min (Fig. 3A). The amount of CC_8R_3 increased to about 20% (of the total radioactivity) within 1 min and then decreased slightly during the ensuing 30 min. In contrast, as

FIG. 3. Identification of intermediates in the assembly process by use of C_8 as a "stopping" reagent. The procedure was similar to those in Fig. 2 except that 100- μ l aliquots were removed at various times and the reactions were "stopped" by the addition of 50 μ l of 40 μ M C_8 . In (A) the initial concentrations were 10 nM of *C and 120 nM of R . In (B) the reaction mixtures contained initially *C at 10 nM and R at 600 nM. The species CC_8R_3 migrated on the gels to a position significantly ahead of C_2R_3 .

shown in Fig. 3B, the rate of formation of C_2R_3 was much less (than in Fig. 3A) when the initial concentration of R was raised to 600 nM, and the principal product formed initially was CC_8R_3 , amounting to about 30% in 1 min. During the following 30 min there was a slight decrease in the amount of CC_8R_3 and a concomitant slow formation of C_2R_3 .

Identification of CR_3 as a Transiently Stable Intermediate During the Assembly Process. Since the kinetic results indicated that CR_3 was formed in appreciable amounts from C and R when the latter was in large excess, we attempted to demonstrate its existence and evaluate its stability by direct physical chemical methods. This was done by using C_{TNF} , since various species containing it could be detected and their $s_{20,w}$ values could be measured with absorption optics (24) and light of 344 nm. As seen in Fig. 4A (pattern 1), 1.5 μ M C_{TNF} had an $s_{20,w}$ value of 6 S, a value only slightly greater than that, 5.8 S, observed for pure C (1). Upon the addition of a stoichiometric amount of R , the C_{TNF} boundary disappeared and, instead, a species having an $s_{20,w}$ value of 12 S was observed.

Rapid mixing of R and C_{TNF} to give concentrations of 200 μ M and 1.5 μ M respectively, led to the formation of two principal components with $s_{20,w}$ values of 9 S and 12.8 S (pattern 2 in Fig. 4A). In different experiments the amount of 9S component varied from 50 to 90%, with about 10% of the material appearing as aggregates. The 9S species was converted to the 12.8S component upon storage of the solution at room temperature. In one experiment the 9S material decreased from 70% of the total at 1 hr after its formation to 50% at 5 hr and 15% at 24 hr. When 1.5 μ M C_{TNF} was mixed with a stoichiometric amount of R and then sedimented in the presence of 200 μ M R , the observed boundary, as seen in Fig. 4A (pattern 3), had an $s_{20,w}$ value of 12.8 S. Thus, the 9S intermediate was

§ The species C_2R_2 and CC_8R_3 had similar electrophoretic mobilities. Hence, their amounts could not be measured separately, and the species identified as CC_8R_3 really represents the sum of C_2R_2 and CC_8R_3 . However, as shown later, the rate of reaction of C_2R_2 with R is extremely rapid and, since the experiments illustrated in Fig. 3 involved excess R , it is unlikely that appreciable concentrations of C_2R_2 could accumulate. Therefore, the species in the position corresponding to C_2R_2 and CC_8R_3 on the gels are likely to be predominantly CC_8R_3 . Further experimentation is needed to eliminate this source of ambiguity.

Fig. 4. Identification of CR_3 by ultracentrifugation. In (A) are shown sedimentation velocity patterns (24 min at 60,000 rpm) with the photoelectric scanner using light of wave length 345 nm. Pattern 1 corresponds to $1.5 \mu M$ C_{TNP} . Pattern 2 was obtained with a solution prepared by rapid mixing of $1.5 \mu M$ C_{TNP} and $200 \mu M$ R. Pattern 3 was obtained with a solution containing the same amounts of C_{TNP} and R, but the solution was prepared by mixing stoichiometric amounts of C_{TNP} and R, and after 5 min excess R was added. The absorbance at 345 nm was 0.15. Since R at these large concentrations had a slight absorbance at 345 nm, it was added to the reference compartment of the double-sector cell. A slight mismatch in the menisci (M) in the second experiment caused the curvature in pattern 2 due to the sedimentation of R. The arrows indicate the positions corresponding to 3S, 6S, 9S, and 12S components. In (B) are shown the results from a sedimentation experiment, 49,000 rpm for 270 min at 0° , in a sucrose gradient varying from 2.5 to 10%. The gradients contained $33 \mu M$ R throughout. The curve indicated by $\bullet$ was obtained with C and R mixed rapidly to give concentrations of $0.4 \mu M$ and $33 \mu M$, respectively. The symbol, $\circ$, designates the results when the subunits were mixed in stoichiometric amounts and then excess R was added 5 min later to give $33 \mu M$. Results are plotted as enzyme activity per ml against fraction number from the meniscus (M). The arrows show positions of 6S, 9S, and 12S species determined from C, aldolase, and ATCase.

formed only when a large excess of R was added directly to C_{TNP} ; moreover, the intermediate had only limited kinetic stability, as shown by its conversion to the species having an $s_{20,w}$ value only slightly greater than that of ATCase.

The intermediate with an $s_{20,w}$ value of about 9 S was also seen as a major component upon rapid mixing of unmodified C ($0.4 \mu M$) and R ($33 \mu M$). This is shown by the results (Fig. 4B) of the sedimentation experiments in a sucrose gra-

Fig. 5. Kinetics of assembly of ATCase from C_2R_2 and *R . The procedure was similar to those in Fig. 2, with the concentrations of C_2R_2 and *R equal to 2.8 nM and 15 nM , respectively, and the "stopping" concentration of R equal to $120 \mu M$. (A) The % of the radioactivity converted to ATCase as a function of time. These results are plotted in (B) as $\alpha \times 10^{-8}$ against time, where α is defined by $\{1/([R] - [C_2R_2])\} \{ \ln[C_2R_2]/([C_2R_2] - [C_2R_3]) - \ln[R]/([R] - [C_2R_3]) \}$. The slope, after correction for the statistical factor, 4, for the number of free sites, gave $1.2 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ for the intrinsic rate constant.

dient containing $33 \mu M$ R. Prior mixing of stoichiometric amounts of C and R, followed 5 min later by the addition of a large excess of R, gave predominantly ATCase.

On the basis of the well-known relationship (25) that $s_{20,w}$ is proportional to the two-thirds power of the molecular weight, $s_{20,w}$ for CR_3 is estimated to be 9.4 S and 9 S from the measured values for C and ATCase, respectively. Thus, the observed value of 9 S for the intermediate is consistent with the interpretation that it has the structure, CR_3 .

Rate of Formation of the c:r Bonding Domain. Since the assembly of ATCase from C and R, as seen in Fig. 1, involves a series of different reactions occurring simultaneously, a kinetic analysis in terms of the formation of the c:r bonding domains is complex. However, the availability of the reasonably stable intermediate, C_2R_2 , permitted a direct measurement of the rate of the relatively simple reaction, $C_2R_2 + R \rightarrow C_2R_3$. Such studies were conducted with *R and C_2R_2 at different molar ratios. When *R was in excess (Fig. 5), C_2R_2 was converted completely to ATCase within 10 min even at very low concentrations of C_2R_2 (2.8 nM) and *R (15 nM). On the assumption that the reaction is irreversible, we plotted the data in terms of the transform of a general second-order rate reaction. Fig. 5 shows that the data from two experiments fit a straight line with a slope corresponding to a rate constant of $1.2 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ for concentrations in terms of free sites. Analogous experiments with an excess of C_2R_2 gave approximately linear plots with a slope of $1.7 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$.

DISCUSSION

The technique described here, though still in a preliminary state of development, shows considerable promise for the study of assembly processes because of its great sensitivity and reasonable precision. Improvements in electrophoretic resolution are needed so that species like C_2R_2 and CC_3R_3 can be separated. If misleading results are to be avoided, it is essential that the rate of dissociation of C from ATCase be so

small that there is no exchange of *C from already formed ATCase when excess C is added as a "chase." Similar considerations apply when excess R is used to "stop" the assembly. These requirements appear to be satisfied since no release of *C or *R was observed from *C_2R_3 or $C_2^*R_3$, respectively, when they were incubated with unlabeled subunits for one week. This stability apparently is achieved without slow annealing of the nascent complexes, since virtually no exchange was detected when unlabeled subunits were added only 5 sec after the formation of *ATCase at protein concentrations such that assembly was complete within 2 sec. C_2R_2 appeared to be similarly resistant to subunit exchange in the assembly experiments even though it does disproportionate under different conditions to give C_2R_3 and free C (15).

Certain aspects of the assembly of ATCase from its subunits are already clear. When C is in excess (relative to R) the intermediate, C_2R_2 , is formed as a principal product, as shown in Fig. 2B. It is likely, but not proved, that C_2R_2 is formed transiently when R is in excess. The material shown as C_2R_2 in Fig. 2A may be largely CR_2 that was then converted to C_2R_2 during the "chase." As expected from the scheme in Fig. 1, the production of ATCase is actually diminished when R is in very large excess (see Fig. 3B). Under these circumstances the dominant species is CR_3 , the existence of which had already been inferred by Chan and Mort (16) from the unusual enzyme kinetic behavior of mixtures containing small amounts of C and a large excess of R. Although as yet no direct determination of the molecular weight has been made, the $s_{20,w}$ value of 9S provides strong evidence that this intermediate has the structure CR_3 .

The intrinsic second-order rate constant, $1 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$, obtained from the reaction between C_2R_2 and R to give ATCase, is only a preliminary value. Further improvements in the preparations and the technique will doubtless lead to some refinement in that value. Perhaps confirmatory evidence can also be obtained from the sequence of reactions leading to CR_3 when a large excess of R is added to C. A mathematical model predicting the concentration of all intermediates in Fig. 1 as a function of time has been developed and subjected to computer analysis (M. Bothwell and H. K. Schachman, in preparation). This model, coupled with the assumption that the rate constant for the rupture of a c:r bond is 10^{-2} sec^{-1} , accounts satisfactorily for the data obtained thus far. With these rate constants it is possible also to account approximately for the kinetics at the higher protein concentrations in the stopped flow experiments.

As yet there is no direct information as to the rate of rupture of a c:r bonding domain. In ATCase all the R subunits are held in place by two such bonding domains and dissociation is extremely slow. With CR_3 , however, it should be possible to measure the rate of dissociation of R relatively directly. Such data, in conjunction with the rate constant for the formation of a c:r bond, would provide valuable information about the strength of these bonding domains. If these rates can be measured in solutions containing different effectors, like substrate analogs and inhibitor, we may be able to assess the role of the c:r bonding domains in mediating the homotropic and heterotropic effects exhibited by ATCase[†].

The relationship of these assembly experiments *in vitro* to the biosynthesis of ATCase has yet to be established. If in *Escherichia coli* under complete repression by uracil there is only one molecule of ATCase per cell, the concentration of enzyme would be about 10^{-7} M . At this level of subunits the assembly of ATCase would be complete in a few seconds. Thus, if the formation of ATCase *in vivo* involves preformed subunits, the assembly via the reactions depicted in Fig. 1 would not represent a rate-limiting process since the synthesis of the polypeptide chains (and perhaps their folding and association to give C and R subunits) would be slower.

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[†] Preliminary observations (I. Gibbons and H. K. Schachman, unpublished) indicate that the rate of dissociation of c:r bonding domains is affected by ligands since the disproportionation of C_2R_2 into C_2R_3 and C was markedly enhanced by the addition of the transition state analog, N-(phosphonacetyl)-L-aspartate (26).