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Aspartate Transcarbamoylase Molecules Lacking One Regulatory Subunit

(allosteric enzymes/protein–protein interactions/subunit assembly)

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ABSTRACT **Reconstitution of aspartate transcarbamoylase (EC 2.1.3.2) from dilute solutions of the isolated regulatory and catalytic subunits, with the latter in large excess, led to the formation of appreciable amounts of a second, stable component in addition to the reconstituted enzyme. The purified component, designated r_4c_6, was found to have a molecular weight about 3×10^4 less than that of the native enzyme, and it combined with isolated regulatory subunit to form aspartate transcarbamoylase. It also combined with *one* succinylated regulatory subunit to form a hybrid species that was identified electrophoretically. These findings indicate that r_4c_6 differs from the native enzyme in that only two (rather than three) regulatory subunits participate in "crosslinking" the two catalytic trimers. The "incomplete" enzyme, r_4c_6, exhibits the characteristic sigmoidal saturation behavior and CTP inhibition of aspartate transcarbamoylase; however these allosteric effects are reduced in extent by about one-third in comparison to the native enzyme and free catalytic subunits. The complex, which may be an intermediate in the assembly and dissociation of the native enzyme, is useful in assessing the role of the various bonding domains responsible for the stability and regulatory properties of the native enzyme.**

Following the discovery that the regulatory enzyme, aspartate transcarbamoylase (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) from *Escherichia coli*, is composed of discrete subunits for catalysis and regulation, each containing a unique polypeptide chain (1), there have been many studies aimed at determining the structure and mechanism of action of the enzyme (2, 3). It is now known that it is composed of six catalytic (c) and six regulatory (r) polypeptide chains (4–7) arranged in a molecule having 2-fold and 3-fold axes of symmetry (8–11). The catalytic chains are organized as trimers (C) both in the intact enzyme and after its dissociation with certain mercurials (5). Although the C subunits show little tendency to associate to form discrete species, they combine readily with free regulatory subunits (R), yielding reconstituted molecules with the unusual physical and kinetic properties of the native enzyme (1, 12). The R subunits, as a result, have been viewed as "crosslinks" that bind the two C subunits in the intact enzyme molecules.

Abbreviations: R, regulatory subunit; C, catalytic subunit; N (subscript), native; S (subscript), succinylated; r, regulatory polypeptide chain; c, catalytic polypeptide chain.

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Various experiments have shown that the r chains are organized as dimers both in the native enzyme and in the R subunit released by treating the enzyme with mercurials (7). In addition, considerations of the weight composition of the enzyme in terms of c and r chains (1, 6, 7) plus hybridization experiments with native C and mixtures of native and succinylated R (13) have led to the conclusion that there are three R dimers in each enzyme molecule. Recent evidence from electron microscopy (14) and x-ray diffraction studies (10, 11) has provided support for a model of the enzyme as a complex containing two catalytic trimers bonded through three regulatory dimers; i.e., R_3C_2 or r_6c_6 (7). According to this model there are six c:c bonding domains linking c chains within the two trimers, three r:r bonding domains between the pairs of r chains in the three dimers, and six r:c bonding domains linking the r and c chains.

In this report we describe a structural variant of aspartate transcarbamoylase, which is found in small amounts in most preparations of enzyme isolated from *E. coli* and which is produced in large amounts when the enzyme is reconstituted from preparations of C and R. As shown below, this complex is an R-deficient aspartate transcarbamoylase composed of two catalytic trimers and only two regulatory dimers; i.e., R_2C_2 or r_4c_6. The existence and properties of this relatively stable complex provide some insight regarding the strength of the bonding domains responsible for the structure and behavior of the native enzyme.

We have investigated the kinetic properties of r_4c_6 in an effort to determined how the R subunits endow the native enzyme with its characteristic allosteric behavior (15, 16). The catalytic activity of aspartate transcarbamoylase varies in a sigmoidal fashion with increasing substrate concentration (homotropic effect), and the enzyme is inhibited by CTP, the end product of the pyrimidine biosynthetic pathway (heterotropic effect). In contrast, C subunits retain all the catalytic activity but lack both regulatory functions (1, 2, 12, 17, 18). The r_4c_6 complex exhibits significant homotropic and heterotropic effects, a result bearing directly on speculations about the structural requirements for allosteric interactions in the native enzyme.

MATERIALS AND METHODS

Enzyme Assay. Aspartate transcarbamoylase activity was measured by the procedure of Porter *et al.* (19) with L-[^{14}C]-aspartate obtained from New England Nuclear Corp. Assays were performed at 30° with "standard imidazole buffer" composed of 50 mM imidazole-imidazole acetate, 2 mM 2-mercaptoethanol, and 0.2 mM Na_2 EDTA at pH 7.0.

Ultracentrifugation. Sedimentation velocity experiments were conducted with a Beckman model E ultracentrifuge at 60,000 rpm. Schlieren patterns were photographed on Metallographic plates which were analyzed on a Gaertner microcomparator. Two samples were measured simultaneously by using paired cells, one of which contained a 1° quartz wedged window to elevate the schlieren pattern and the other of which contained conventional windows with parallel surfaces. Difference sedimentation equilibrium experiments were conducted with Rayleigh optics according to the method of Springer *et al.* (20).

Electrophoresis. Polyacrylamide disc gels were used as described by Ornstein (21) and Davis (22). The gels were prepared according to the "Tris system" of Jovin *et al.* (23). Zone electrophoresis was performed with a Beckman model R-101 microzone cell and cellulose acetate membranes (5). The buffer was 25 mM Tris–Tris chloride at pH 8.0, and 300 V were applied for 20–30 min.

Preparation of Enzyme and Subunits. Aspartate transcarbamoylase from *E. coli* was prepared by the method of Gerhart and Holoubek (12). R and C were prepared by treating the enzyme with the mercurial, neohydrin [1-(3-chloromercuri-2-methoxypropyl)-urea] followed by chromatography on DEAE–cellulose (24, 18). After the fractions of R were collected, 2-mercaptoethanol was added to a concentration of 10 mM; this was followed by the addition, 10% by volume, of a solution containing 0.5 M imidazole–imidazole chloride (pH 7.5)–20 mM zinc acetate. Solutions of each subunit were then dialyzed against 3.6 M $(NH_4)_2SO_4$–10 mM 2-mercaptoethanol for concentration and storage. Protein concentrations were determined from their absorbance at 280 nm, from known extinction coefficients (7).

Succinylated regulatory subunits (R_S) were prepared by reaction of the native enzyme with succinic anhydride followed by dissociation of the modified enzyme by treatment with neohydrin; R_S was purified by chromatography on DEAE–cellulose (G. M. Nagel and H. K. Schachman, submitted to *Biochemistry*).

RESULTS

Preparation of r_4c_6. As shown in Fig. 1*a*, many preparations of aspartate transcarbamoylase contain a small amount of a component that migrates more rapidly in electrophoresis in polyacrylamide gels§. This minor component is not detected in solutions of either subunit alone (see Figs. 1*e* and 1*f*), but it is observed in reconstitution experiments when C is in excess relative to R. The rapidly migrating component was shown to contain C in experiments with catalytic subunits labeled with ^{125}I (see Figs. 5 and 7 in ref. 25). As seen in Fig. 1*b*, the addition of free R to preparations of the native enzyme led to the disappearance of the faster-migrating component. These preliminary observations indicated that the rapidly migrating component contained the same subunits as the native enzyme. In order to permit further characterization of the faster component, we initiated efforts to obtain it in purified form.

Significant yields of r_4c_6 were obtained in reconstitution experiments when C was in large excess and both types of subunits were at low concentration. A typical experiment involved the addition of 10 mg of R in 150 ml of 25 mM Tris–Tris·chloride, 10 mM 2-mercaptoethanol, and 0.2 mM zinc acetate (pH 8.0) to 100 mg of C in 10–20 ml of the same buffer. After incubation at 30° for 30 min the solution was concentrated by filtration under N_2, dialyzed, and analyzed by disc-gel electrophoresis. Such experiments yielded about 20 mg of reconstituted aspartate transcarbamoylase, 10 mg of r_4c_6, and 80 mg of free C. Purification of r_4c_6 required the removal of C by gel filtration on Sephadex G-200 followed by chromatography on DEAE–Sephadex. Fig. 2 shows the elution profile

§ The slowly migrating components represent aggregates such as dimers and related species.

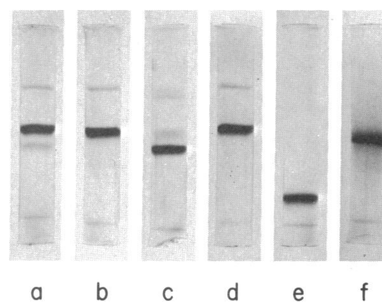

FIG. 1. Polyacrylamide gel electrophoresis of aspartate transcarbamoylase, r_4c_6, and controls. Sample *a* represents purified, native aspartate transcarbamoylase containing a small amount of a more rapidly migrating component. Addition of R to that preparation followed by electrophoresis gave the pattern shown in *b*. Partially purified r_4c_6 gave the pattern in *c*. Upon the addition of R to that sample, the pattern in *d* was obtained. The patterns in *e* and *f* were obtained with purified C and R, respectively.

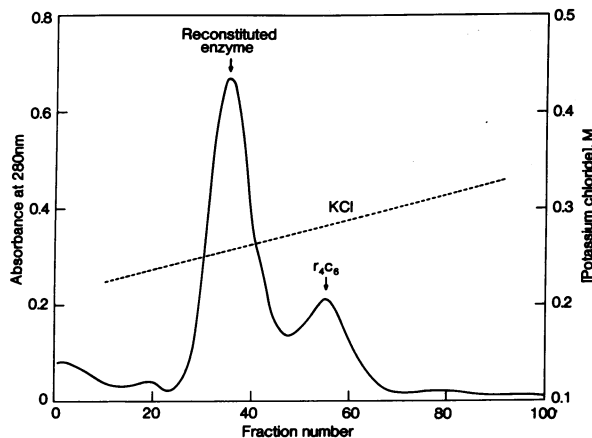

FIG. 2. Purification of r_4c_6. Both aspartate transcarbamoylase and r_4c_6 were formed in a reconstitution experiment with a large excess of C relative to R. The products of the reaction, after 30 min of incubation at 30°, were dialyzed against 0.1 M Tris–Tris·chloride at pH 8, and the protein was concentrated by filtration under N_2. Excess C was removed by gel filtration on Sephadex G-200. The pooled fractions representing native and incomplete enzyme were concentrated and passed again through a Sephadex G-200 column to remove the contaminating C, and the pooled fractions containing aspartate transcarbamoylase and r_4c_6 were concentrated and dialyzed against 50 mM Tris–Tris·chloride, 2 mM 2-mercaptoethanol, 0.22 M KCl at pH 7.5. The protein was adsorbed on an equilibrated DEAE–Sephadex column; elution was performed with 500 ml of buffered solution containing a gradient of KCl varying from 0.22 M to 0.48 M. The *solid curve* represents the absorbances of the various fractions; the *dashed curve* gives the KCl concentration.

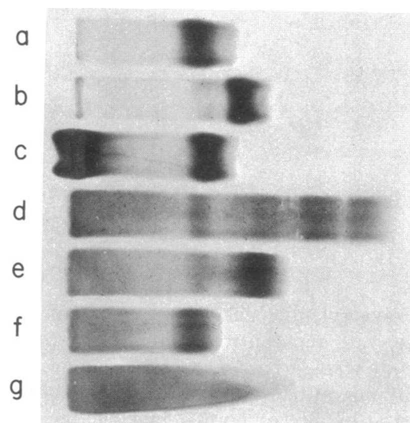

FIG. 3. Reaction of r_4c_6 with succinylated regulatory subunits, R_S. Electrophoresis on cellulose acetate membranes was performed as described in *Methods*. Samples *a* and *b* represent native aspartate transcarbamoylase and r_4c_6, respectively. The pattern in *c* was obtained on a sample of r_4c_6 to which R_N was added before electrophoresis. Sample *d* represents the four-membered hybrid set formed by reconstitution of enzyme-like molecules from C and equal amounts of R_N and R_S. The patterns in *e* and *f* were obtained by addition of R_S to r_4c_6 and aspartate transcarbamoylase, respectively. Sample *g* represents the pattern for R_S.

of the reconstituted enzyme and r_4c_6 from the DEAE–Sephadex column. Pooling of fractions 48 through 70, followed by concentration of the solution and rechromatography with a more shallow salt gradient led to a preparation (Fig. 1*c*) containing approximately 95% r_4c_6 and 5% aspartate transcarbamoylase. Addition of purified R to this sample, as shown in Fig. 1*d*, led to the disappearance of the major (more rapidly migrating) component with the concomitant formation of a large amount of aspartate transcarbamylase.‖

Molecular Weight of r_4c_6. Sedimentation studies were conducted on r_4c_6 in order to determine its composition in terms of C and R, which have molecular weights of 1.0×10^5 and 3.4×10^4, respectively (6, 7). The sedimentation coefficient of r_4c_6 was found to be 7% less than that of the native enzyme. This value corresponds to a molecular weight difference of 3×10^4 for spherical protein molecules (26). This calculated difference in molecular weight would be slightly larger if the frictional coefficient of the intact enzyme is larger than that of r_4c_6.

Difference sedimentation equilibrium measurements showed that r_4c_6 had a molecular weight 3.2×10^4 less than that of aspartate transcarbamoylase (M. Springer and H. G. Schachman, in preparation).

Molecular weights were also obtained from the mobilities of different proteins on polyacrylamide gels of various porosity (27). This technique, based on the native enzyme and C as standards, gave 2.8×10^5 for the molecular weight of r_4c_6 [compared to 3.1×10^5 for the native enzyme (6, 7)].

Number of Missing Regulatory Polypeptide Chains in r_4c_6. Although the molecular weight and electrophoresis experiments indicate that the incomplete enzyme lacks some regulatory chains, they do not warrant a conclusion as to the number of missing chains. Hence we investigated the reaction of succinylated regulatory subunits, R_S, with the incomplete enzyme, since the composition and properties of various complexes of R_S and R_N with C are known from hybridization experiments (ref. 13; G. M. Nagel and H. K. Schachman, submitted to *Biochemistry*). These studies provided a four-membered hybrid set of enzyme-like molecules that had the composition $R_NR_NR_N(C)_2$, $R_NR_NR_S(C)_2$, $R_NR_SR_S(C)_2$, and $R_SR_SR_S(C)_2$.

Figs. 3*a* and 3*b* show electrophoresis patterns of native enzyme and r_4c_6, respectively. Addition of R_N to the latter led to the formation of aspartate transcarbamoylase, as seen in Fig. 3*c*. The four-membered hybrid set formed by the rapid addition of R_N and R_S to a solution of C is shown in Fig. 3*d*. Upon the addition of R_S to r_4c_6 (as seen in Fig. 3*e*), a hybrid molecule is formed having the same mobility as the second member of the hybrid set obtained in the reconstitution reaction with the isolated subunits. Hence, its composition is $R_NR_NR_S(C)_2$. This hybrid is not the product of an exchange reaction involving regulatory subunits; as seen in Fig. 3*f*, the addition of R_S to native enzyme produced no hybrids. Moreover, the hybrid, $R_NR_NR_S(C)_2$, proved stable as judged by the absence of the other species which would have formed through disproportionation reactions.

Homotropic and Heterotropic Interactions Exhibited by r_4c_6. In terms of structure, r_4c_6 can be viewed as an intermediate between native aspartate transcarbamoylase, an allosteric enzyme, and free C, which exhibits no allosteric behavior. Would such an incomplete molecule exhibit the kinetic properties of an allosteric enzyme and could we assess the role of the regulatory subunits in mediating allosteric effects?

Initial experiments with r_4c_6 extracted from polyacrylamide gels after electrophoretic separation showed that it possessed considerable enzyme activity and sensitivity to the feedback inhibitor, CTP. Quantitative studies were performed on the column-purified material and the results are shown in Fig. 4, along with those for the native enzyme and free C. As with native aspartate transcarbamoylase, the initial velocity of the reaction catalyzed by r_4c_6 varies in a sigmoidal fashion with the concentration of the substrate, aspartate. In contrast, this dependence of initial velocity on substrate concentration is hyperbolic with C. This difference between the native enzyme and r_4c_6, on the one hand, and free C on the other is emphasized in Fig. 4*b* where the kinetic data are plotted as the initial velocity divided by the aspartate concentration against the initial velocity (28). The curvature for the native and incomplete enzyme is indicative of homotropic, cooperative interactions, whereas the linarity for the catalytic subunits is characteristic of enzymes with either one or several independent active sites. As seen in Table 1, r_4c_6 exhibits both homotropic and heterotropic effects, although the Hill coefficient and the CTP inhibition are less than those for aspartate transcarbamoylase (data for both the native and reconstituted enzyme are given since the latter generally shows slightly less allosteric behavior). However, the maximal velocity of r_4c_6 is the same as that of the native enzyme and strikingly different from that of free C.

Addition of free R to r_4c_6 led to an increase in the Hill coefficient to 1.6, a value equal to that found for reconstituted aspartate transcarbamoylase. In contrast, the addition of R_S

‖ Figs. 1*c* and 1*d* show that the faster of the aggregated species is converted into the other aggregate upon the addition of R. This observation indicates that R-deficient aggregates are formed in the reconstitution process.

TABLE 1. *Kinetic properties of r_4c_6 and related species*

	Hill coefficient	Inhibition by CTP* (%)	Maximal velocity
Catalytic subunit	1.0	7	33†
Native enzyme	1.7	60	16†
Reconstituted enzyme	1.6	55	16
r_4c_6	1.4	36	16†
$r_4c_6 + R_N$	1.6	—	16
$r_4c_6 + R_S$	1.4	—	15

* The percent inhibition of catalytic activity by 0.5 mM CTP at saturating amounts of carbamoyl phosphate (4 mM) and at 5 mM aspartate.

† These values, obtained from the intercepts in Fig. 4*b*, represent μmol of carbamoyl aspartate per hr per μg of catalytic subunit protein. Native enzyme contains 64% catalytic subunit and r_4c_6 contains 72% catalytic subunit by weight.

to r_4c_6 caused no change in the Hill coefficient of 1.4 (see Table 1).

DISCUSSION

Structure of r_4c_6. As shown in Fig. 1, r_4c_6 combines with free R to form a complex having the electrophoretic mobility of aspartate transcarbamoylase. In addition, r_4c_6 has a molecular weight about 3×10^4 less than that of the native enzyme. Since r chains have a molecular weight of 1.7×10^4, these findings indicate that r_4c_6 has two less r chains than the native enzyme. Further support for this view came from examining the reaction between R_S and r_4c_6. The complex formed in this way corresponds to the hybrid molecule, $R_NR_NR_S(C)_2$. Hence we conclude that r_4c_6 lacks two r chains as compared to native aspartate transcarbamoylase.

Are the two missing r chains from the same subunit or are they from two different subunits? Several considerations favor a model lacking one regulatory dimer. Such a structure would retain two r:r and four r:c bonding domains and might be expected to be reasonably stable as compared to the native enzyme. In contrast, if the two missing r chains were from different R subunits the linking of R and C in the complex would be dependent on only one r:r and two r:c bonding domains. This type of structure would be much less stable; if it could exist we might expect that a complex lacking four r chains would be detected. No such species has been observed; indeed the studies with R_S show that only one subunit combines with r_4c_6. Finally, the hybridization experiments on the reconstitution of enzyme-like molecules from R_N and R_S with C (G. M. Nagel and H. K. Schachman, submitted to *Biochemistry*) indicate that the combining unit is a dimer rather than single chains.

What is the possibility that r_4c_6 has a structure totally unrelated to that of the native enzyme? This seems unlikely since the products formed in the reaction of r_4c_6 with either R_N or R_S are reasonably homogeneous and correspond to aspartate transcarbamoylase and one of its known hybrids, respectively. These findings indicate that the structure of r_4c_6 is similar to that of the native enzyme. Further evidence must await studies with covalently, crosslinked R subunits (7).

Storage of r_4c_6 for several weeks in Tris buffer led to its partial conversion to a mixture of r_6c_6 and free C. In buffers of low

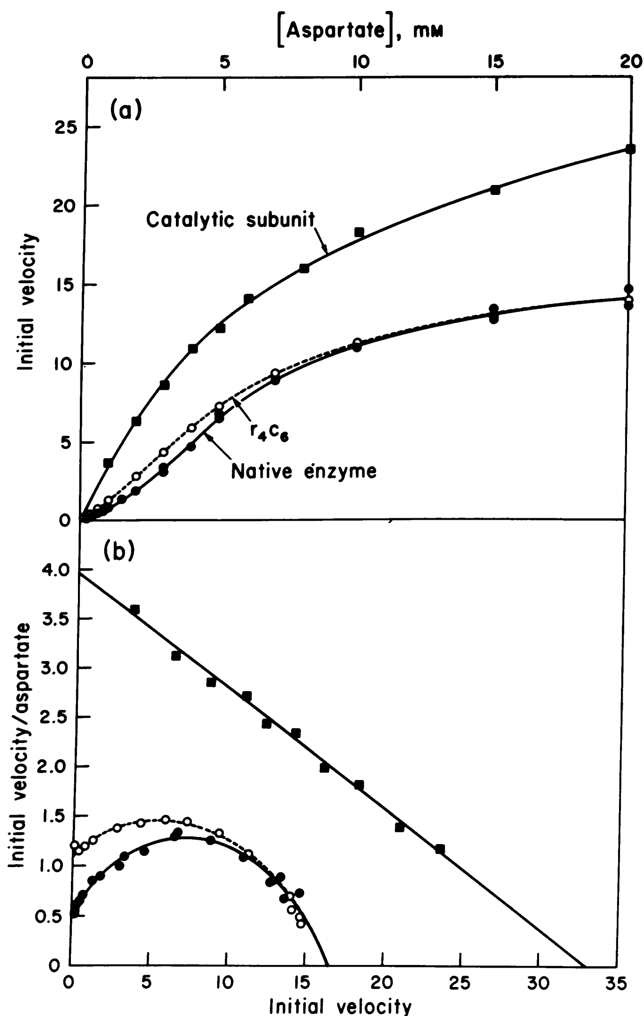

FIG. 4. Kinetic properties of r_4c_6 and related species. Assays were performed at 30° with solutions containing 4 mM carbamoyl phosphate and various amounts of aspartate. Since the three proteins contain different amounts of enzymically active protein, the reaction velocities were normalized to give μmol of carbamoyl aspartate per hr per μg of catalytic protein. Saturation curves are given in (*a*) and the data are plotted in (*b*) as initial velocity/aspartate against initial velocity (28). Results for C are designated by ■, for native enzyme by ●, and for r_4c_6 by O.

ionic strength** this disproportionation was markedly accelerated, leading to the almost complete disappearance of r_4c_6. Thus it seems that r_4c_6 is less stable than native enzyme and that kinetic factors must be involved in the formation of r_4c_6 from R and C when the latter is present in large excess. Its appearance as a contaminant in preparations of native enzyme may be attributable in part to the heat step in the purification (12). Biosynthetic factors such as insufficient amounts of R or proteolysis of R in derepressed cells may also be implicated. Further studies of the formation and stability of r_4c_6 likely will be useful in evaluating the strength of the various bonding domains and in determining its possible role

** Because of the disproportionation of r_4c_6 in the buffers of low ionic strength used for electron microscopy (14), no satisfactory micrographs have been obtained.

as a stable intermediate in the assembly and dissociation of the native enzyme††.

Allosteric Properties of r_4c_6. As seen in Fig. 4 and Table 1, r_4c_6 differs markedly from free C in its kinetic behavior; rather it displays the homotropic and heterotropic effects characteristic of native enzyme. These effects are reduced, however, to approximately $^2/_3$ those of aspartate transcarbamoylase as compared to free C‡‡.

It is possible that the putative constrained and relaxed states (16) of the enzyme are altered when one R is missing and that the reduction of both the Hill coefficient and the CTP inhibition is due to a general effect on the entire molecule affecting the equilibrium between these states and/or the various affinities for ligands. Alternatively, two of the c chains in r_4c_6 may not be capable of participating in the allosteric interactions because they are not bonded to r chains. The observation that r_4c_6 displays allosteric interactions approximately in proportion to the number of regulatory subunits in the complex leads us to consider whether direct interactions between c and r chains are essential for the mediation of cooperativity and inhibition. Perhaps cooperativity and inhibition are dependent upon a combination of at least four functional c and r chains (c:r:r:c) bonded to each other from one catalytic subunit to that beneath it. In contrast it should be noted that the maximal velocity observed for r_4c_6 indicates that catalytic chains not bonded to regulatory chains exhibit the behavior of chains in the native enzyme and not those of free catalytic subunits.

Recently Warren *et al.* (10) have suggested that regulation in aspartate transcarbamylase is achieved through changes in the accessibility of substrates to the central cavity containing the six active sites. The observations on r_4c_6 are particularly relevant in considerations of this proposal. If restriction to access of substrates to the internal cavity were a major factor in the regulatory mechanism, the loss of one R subunit from the enzyme should be accompanied by a marked change in allosteric behavior. Moreover, we would expect $R_NR_NR_S(C)_2$ and r_4c_6 to differ significantly since the former would have a shielded cavity and the latter would permit access through an entire side of the molecule. No such difference is observed; the allosteric properties of the hybrid containing one nonfunctional regulatory subunit are nearly identical to those for the incomplete enzyme (G. M. Nagel and H. K. Schachman, submitted to *Biochemistry*). We consider it more likely that allosteric effects require direct regulatory–catalytic chain interactions and changes in them as a result of alterations in the conformations of the chains upon the binding of ligands.

†† Recently an intermediate has been detected during the tryptic digestion of aspartate transcarbamoylase (29). Although the composition of this component has not been established, it should be noted that it is similar to r_4c_6 in sedimentation coefficient, in electrophoretic mobility on polyacrylamide gels, and in kinetic behavior.

‡‡ Jacobson and Stark (30) independently have isolated R-deficient enzyme molecules and showed that their kinetic properties differed from those of the native enzyme. We are indebted to them for communicating these results before publication and for valuable discussions about the structure and behavior of r_4c_6.

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