Cooperative Interactions in Aspartate Transcarbamoylase. 1. Hybrids Composed of Native and Chemically Inactivated Catalytic Polypeptide Chains

(Allosteric enzymes/cooperativity/reversible charge modification/chromatographic handle)

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ABSTRACT Hybrids of aspartate transcarbamoylase (EC 2.1.3.2; carbamoylphosphate:l-aspartate carbamoyltransferase) from Escherichia coli containing native (active) and pyridoxylated [inactive] catalytic polypeptide chains were constructed by a procedure involving the reversible acylation of amino groups with 3,4,5,6-tetrahydrophthaloyl anhydride. This technique exploited the charges contributed by the tetrahydrophthaloyl groups as a "chromatographic handle" for separating the various species. Enzyme-like molecules containing one fully active and one inactive catalytic subunit showed cooperative kinetic behavior, considerable inhibition by CTP, and a substantially increased apparent Kₐ compared to the native enzyme. Similar properties were observed for an intrasubunit hybrid containing one inactive catalytic polypeptide chain in each subunit. The cooperative inter- and intra-subunit hybrids also exhibited conformational changes similar to those found for the native enzyme upon the addition of stereospecific ligands. These observations, taken together with data for other complexes of catalytic and regulatory subunits, illustrate the importance of the architecture of aspartate transcarbamoylase and the quaternary constraint stemming from the subunit interaction.

Following the discovery (1) that the regulatory enzyme, aspartate transcarbamoylase (ATCase) (EC 2.1.3.2; carbamoylphosphate:l-aspartate carbamoyltransferase) from Escherichia coli, is subject to feedback inhibition by CTP and to activation by ATP, there has been much interest in understanding how these effects are mediated by the protein (2, 3). Similarly, effort has been directed toward developing a molecular mechanism for the observed cooperative (sigmoidal) dependence of enzyme activity on the concentration of the substrate, aspartate (1). These two characteristics of allosteric enzymes, known as heterotropic and homotropic effects, are attributed generally to subunit interactions which permit the molecules to assume different conformations (4, 5).

Abbreviations: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; c, catalytic polypeptide chain; r, regulatory polypeptide chain; x (subscript), native subunit; a (subscript), native chain; s (subscript), succinylated subunit; p (subscript), pyridoxylated subunit; p (subscript), pyridoxylated chain; t (subscript), pyridoxylated and tetrahydrophthaloylated subunit; p₄ (subscript), pyridoxylated and tetrahydrophthaloylated chain; THPA, 3,4,5,6-tetrahydrophthalic anhydride; THP-, tetrahydrophthaloyl; [R], normal complement of native R in ATCase; [R]; Cₐ[R], ATCase; Cₐ[R], ATCase-like molecule lacking one R; CₐCₐ[R]; inter-subunit hybrid containing one native catalytic subunit (Cₐ) and one pyridoxylated catalytic subunit (Cₐ); and the normal complement of R; CₐCₐ[R], inter-subunit hybrid containing two catalytic subunits each of which has two native and one pyridoxylated chain and the normal complement of R.

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Several types of subunit interactions are implicated in stabilizing ATCase as an oligomer (6) composed of six catalytic (c) and six regulatory (r) polypeptide chains (7-9) organized as two catalytic (C) and three regulatory (R) subunits (9-12). Much evidence (13) has been accumulated in support of a model for ATCase as a complex of two C trimers bridged by three R dimers, i.e., CₐR₃. In this structure there are six c:R domains linking the c chains in each of the two C trimers; three r:R domains linking the r chains in the three R dimers, and six c:r domains linking the c and r chains (12, 18). Free C subunits are devoid of cooperativity and inhibition (2, 6, 14) as is the species CₐR₃ (15), which is transiently observed only in the presence of a large excess of R (18). In contrast, the stable R-deficient species, CₐR₂ (17, 18), exhibits both homotropic and heterotropic effects, although their extent is reduced compared to the native enzyme (18).

One systematic approach to an understanding of the structural requirements for cooperativity and feedback inhibition in ATCase involves the construction of hybrid molecules containing modified subunits (9, 10). Can cooperativity and inhibition be obtained with hybrids containing one active (native) C and one inactive C and three R subunits? Would a hybrid containing active and inactive C chains in each C subunit (along with three native R subunits) exhibit allosteric behavior? As shown below, both types of ATCase-like molecules have been constructed, and they exhibit kinetic and physical properties similar to those of the native enzyme.

Inactivation of the C subunits was achieved specifically by reaction of the protein with pyridoxyl 5'-phosphate followed by reduction of the Schiff base with NaBH₄ (19). Since reconstituted ATCase-like molecules containing native and pyridoxylated chains did not differ sufficiently from native enzyme in electrophoretic and chromatographic behavior to permit isolation of the desired species, we introduced additional charged groups by reacting the inactive derivative, Cp, with the "reversible" anhydride, THPA (20). The resulting THP-groups serve as a "chromatographic handle"; after the appropriate hybridization and fractionation these groups were removed readily at pH 6.0. In this way CₐCₐ[R] and CₐCₐ[R] were obtained in purified form.

† Preliminary studies (13, 21, 22) with hybrids containing native and succinylated catalytic chains indicated that cooperativity could be obtained in ATCase-like species containing active chains in only one subunit. This cooperativity is now thought to be attributable to aggregates, since more recent preparations of purified hybrids containing extensively succinylated (inactive) chains invariably exhibit no cooperativity. In order to avoid the large net negative charge resulting from the acylation we subsequently turned to a procedure involving specific inactivation and reversible acylation of the amino groups (20).
MATERIALS AND METHODS

ATCase, Cn and R were prepared by procedures described earlier (23). The enzyme was assayed by the method of Porter et al. (24). Pyridoxylation of Cn with pyridoxal 5'-phosphate followed by reduction of the Schiff base with NaBH₄ was performed according to the technique of Greenwell et al. (19). The resulting derivative, Cₚ, had 1.2 residues of pyridoxamine 5'-phosphate per c chain and retained about 2% of the enzymatic activity of Cn. Pyridoxylated derivatives were stored in the dark except for brief exposure during experimental manipulations.

Acylation with THPA was performed at pH 8.2 by the addition of the THPA solution (0.5 M in dioxane) to the protein (5 mg/ml) in 50 mM K₂HPO₄, 2 mM EDTA, and 2 mM β-mercaptoethanol. The molar ratio of THPA to lysyl residues was 0.6 and the reaction, as judged by the attainment of a constant pH (about 7.7), was complete within about 20 min. The resulting derivative (with 30-40% of the amino groups acylated) was dialyzed against 50 mM Tris·HCl, 2 mM EDTA, 2 mM β-mercaptoethanol at pH 8.25 in order to remove the tetrahydrophthalic acid. THP-proteins were maintained at 0° and pH 8.4 except for the subsequent chromatography. Desalting sufficient to remove more than 95% of the THP-groups was performed by incubating the protein at room temperature for 1–2 days in a 50 mM potassium phosphate buffer at pH 6.9 by the procedure of I. Gibbons and H. K. Schachman (in preparation).

The inter-subunit hybrid set, comprising CNCN[R], CNCPₜ[R] and CPₜCPₜ[R], was formed by mixing CN and CPₜ and then adding R (9). The three species were separated by DEAE-Sephadex chromatography (21). Desalting of the acylated species produced CNCP[R] and CPₜCPₜ[R]. Preparation and purification of the intra-subunit hybrid, Cₙₙₙ[R], were achieved essentially according to the methods of Meighen et al. (9) and Figiet (21), with CN and CPₜ as the parental species. This procedure involved dissociation of a mixture of the two proteins at 1–2 mg/ml in 6.5 M urea and 0.1 M dithiothreitol at 0° for 15 min followed by reconstitution through a rapid 10-fold dilution and dialysis against 50 mM Tris·HCl, 2 mM EDTA, 2 mM β-mercaptoethanol at pH 8.25. Chromatographic fractionation yielded Cₙₙ[R], which was desalting to give Cₙ[R] and then reacted with excess R. The resulting Cₙ[R] was then separated from the excess R by chromatography (9).

Polyacrylamide gel electrophoresis with 5% gels was conducted with the buffer system of Jovin et al. (25). Zone electrophoresis was performed on cellulose phosphate strips (Gelman Sephaphore) in a Microzone Electrophoresis Cell (Beckman Spino) at 250 V for 20 min. The buffer was 25 mM Tris·HCl, 2 mM EDTA, pH 8.0. Measurements of the change in sedimentation coefficients, ΔS/η, upon the addition of ligands were made with a Beckman model E ultracentrifuge (26).

RESULTS

Preparation of Cₙ[R]. The production of the hybrid, CNCPₜ[R], followed by its conversion to CNCP[R], is illustrated in Fig. 1. As seen in the electrophoresis patterns, CN and CPₜ have significantly different mobilities. Most of the CPₜ is reconstituble to give ATCase-like species; the third and seventh patterns represent the inter-subunit hybrid set containing three well-resolved species. These were separated by DEAE-Sephadex chromatography, as shown in Fig. 2.

A small amount of unreconstituted CPₜ[R] is observed at the trailing edge of the CPₜCPₜ[R] peak. The individual fractions representing each of the different species were pooled and identified by electrophoresis as CNCN[R], CNCPₜ[R] and CPₜCPₜ[R] in patterns 4, 5, and 6 in Fig. 1. After removal of the THP-groups the CNCP[R] hybrid and CPₜ[R] were electrophoretically similar to native ATCase. The pattern for CNCP[R] is shown at the bottom of Fig. 1.

Polyacrylamide gel electrophoresis revealed the presence of small amounts (<2%) of R-deficient ATCase (18) and aggregates (12%) in the hybrid preparations. These species were usually found in similar amounts in preparations of reconstituted ATCase.

Preparation of Cₙₙ[R]. Reconstitution of a urea-denatured mixture of CN and CPₜ gave the intra-subunit hybrid set. Chromatographic fractionation of this hybrid set on DEAE-Sephadex yielded the individual components, Cₙₙ[R], Cₙₙ[R], Cₙₙ[R], and Cₙₙ[R]. Removal of the THP-groups from Cₙₙ[R] produced Cₙ[R], which was then mixed with excess R and subjected to chromatography on DEAE-Sephadex to give a preparation of Cₙ[R] containing about 16% aggregates (as judged by polyacrylamide gel electrophoresis) and a very small amount of R-deficient species.
Sephadex chromatography. A linear gradient (conductivities mixing aspartate and Tris-HCl, pH 7.5, containing 0.2 M KCl and 2 mM EDTA) was formed at 20 ml/hr with a linear gradient of KCl varying from 0.2 M to 0.8 M in the same buffer (conductivities are shown at the bottom of the figure). Fractions of 2.0 ml were collected up to 220 ml.

**Enzymic and Physical Properties of the Hybrids.** Fig. 3 shows the kinetic behavior of C_{n}C_{p}[R] along with that of C_{n}C_{n}[R]. As with native ATCase, the specific activity, measured by the velocity of the reaction catalyzed by both C_{n}C_{p}[R] and the reconstituted C_{n}C_{n}[R], varies in a sigmoidal fashion with the concentration of the substrate, aspartate (Fig. 3a). The cooperativity is emphasized in Fig. 3b, where the kinetic data are plotted as specific activity divided by the aspartate concentration as a function of the specific activity (27). Both sets of data show marked curvature with maxima, as contrasted to

![Graph](https://example.com/graph1.png)

Fig. 2. Resolution of the inter-subunit hybrid set by DEAE-Sephadex chromatography. The hybrid set was formed by mixing 31 mg C_{n} and 18 mg C_{p}, and then adding 26 mg R. Fractionation was performed on a 30 × 1.5-em column equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.2 M KCl and 2 mM EDTA. The column was eluted at 20 ml/hr with a linear gradient of KCl varying from 0.2 M to 0.8 M in the same buffer (conductivities are shown at the bottom of the figure). Fractions of 2.0 ml were collected up to 220 ml.

![Graph](https://example.com/graph2.png)

Fig. 3. Kinetics of the hybrid, C_{n}C_{p}[R], and the reconstituted enzyme, C_{n}C_{n}[R]. Assays were performed at 30° in 50 mM imidazole-acetate buffer, pH 7.0, containing 0.2 mM EDTA. The concentration of carbamoylphosphate was 4 mM and the aspartate concentration (mM) was varied. The specific activity is given nmol of carbamoyl aspartate formed per hr/μg of protein. (a) The saturation curve of specific activity versus aspartate concentration. (b) The data plotted as specific activity/aspartate concentration versus specific activity. Values for C_{n}C_{n}[R] are designated by ◦ and for C_{n}C_{p}[R], by O.

<table>
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<tr>
<th>Species</th>
<th>V_{max}*</th>
<th>pH†</th>
<th>K_{m}‡</th>
<th>CarbP +</th>
<th>Succ</th>
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<td>71</td>
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* Values relative to C_{n}C_{n}[R], (V_{max} = 11 μmol of carbamoyl aspartate per hr/μg, a value almost identical to that of native enzyme).
† Value of Hill coefficient for the substrate aspartate, measured between 10 and 50% of V_{max}.
‡ Concentration of the substrate aspartate required for half maximal velocity.
§ CTP concentration was 0.5 mM and the inhibition was measured at 3 mM aspartate.
¶ Values of changes in sedimentation coefficient, Δs/3, were obtained with 2 mM carbamoylphosphate (CarbP) in one cell compared to an equivalent amount of phosphate in the reference solution; when succinate (Succ) was added (2 mM) in addition to carbamoylphosphate, glutarate and phosphate were added to the reference solution. The solvent was 40 mM phosphate (K +), pH 7.0, containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol. Δs/3 for identical solutions was ±0.3%. (At pH 7.0 the THP-species lose some (<10%) of the THP-groups during the determination.)
‖ Values represent the calculated differences between those measured for (carbamoylphosphate + succinate) and carbamoylphosphate alone.

** Activity measured at 30 mM aspartate.

The linear plots observed with non-cooperative species like C_{n}, which contains three independent active sites (2, 6, 14). Figure 3 also shows, as expected on the basis of the number of catalytically active chains in the two species, that the maximum specific activity of C_{n}C_{p}[R] is one-half that of C_{n}C_{n} [R].

Table 1 summarizes the kinetic and physical properties of the various hybrids and of the corresponding parental species. Both C_{n}C_{p}C_{p} [R] and C_{n}C_{p} [R] were virtually inactive compared to C_{n}C_{n} [R]. Neither of these ATCase-like derivatives showed the conformational change characteristic of the native enzyme (26). This is seen by the small changes in the sedimentation coefficient, Δs/3; upon the addition of the aspartate analog succinate to solutions of the proteins containing the substrate carbamoylphosphate.

The hybrids C_{n}C_{p}C_{p} [R] and C_{n}C_{p} [R] had specific activities (as indicated by the maximal velocity, V_{max}) corresponding to 42% and 52%, respectively, of that of native ATCase. Whereas C_{p}C_{p}C_{p} [R] showed no cooperativity (Hill coefficient, n_H = 1.0) and only slight inhibition by CTP (23%), the removal of the THP-groups to give C_{p}C_{p} [R] led to considerable cooperativity (n_H = 1.38) and CTP inhibition (72%). The value of the apparent K_{m} for C_{p}C_{p} [R] was significantly higher than those for both C_{n}C_{n} [R] and C_{p}C_{p}C_{p} [R]. As seen by the values of Δs/3, the cooperative hybrid, C_{p}C_{p} [R],
exhibited a small conformational change upon the addition of carbamoylphosphate and a much larger change, characteristic of the native enzyme (26), upon the subsequent addition of succinate. In contrast, for the non-cooperative hybrid, CnP\_Cp\_R, the value of \( \Delta S/3 \) was much larger upon the addition of carbamoylphosphate, and there was a much smaller subsequent change when succinate was added.

Table 1 also shows that the intra-subunit hybrid C\_nnp-C\_nnp\_R had a specific activity corresponding to the number of catalytically active polypeptide chains. Moreover, it exhibited kinetic and physical behavior similar to that of the inter-subunit hybrid, C\_nCp\_R.

**Evaluation of Experimental Hazards.** Reconstitution of ATCase from isolated subunits invariably produces small amounts of aggregates and R-deficient molecules, CnR2 (6, 18). Since DEAE-Sephadex chromatography fails to resolve “monomers” from higher aggregates having the same ratio of charge to mass, the preparations of the hybrid, C\_nCp\_R, may be contaminated with a mixed “dimer” comprising both parental types. Assuming that the kinetic behavior of the aggregates can be represented by the sum of their component parts, we conclude that the contribution of aggregates to the kinetic properties of C\_nCp\_R is likely to be very small. Moreover, removal of aggregates from C\_nCp\_R by chromatography on Sephadex G-200 resulted in no significant change in the kinetic parameters. Although R-deficient ATCase molecules are not fully resolved from C\_nCp\_R by the chromatographic procedure, their amount is too small to affect the kinetics of the resulting C\_nCp\_R. Aggregates and R-deficient molecules in the preparation of C\_nnp-C\_nnp\_R probably contribute no complication, since they are derived from only one type of catalytic subunit. With hybrids like C\_nCp\_R and C\_nCp\_R, there is the risk that disproportionation could lead to the formation of the parental species. However, the gradual removal of the labile THP-groups was accompanied by a progressive change in the electrophoretic mobility of all molecules (without the formation of the parental species); hence we conclude that C\_nCp\_R \[RI \] did not disproportionate. In addition, the observed kinetic properties (apparent \( K_m \)) of the hybrids differed from those expected for the corresponding one-to-one mixture of parental species.

Prolonged exposure (24 hr) of C\_n to light from a fluorescent lamp led to considerable restoration (up to about 70%) of enzyme activity. Hence, manipulations with pyridoxylated derivatives were conducted in darkened rooms and vessels were covered with aluminum foil. In this way, reactivation was minimized, as shown by the results with C\_nCp\_R, which was virtually inactive (4% of native ATCase).

**DISCUSSION**

As seen in Fig. 3 and Table 1, the inter- and intra-subunit hybrids containing active and inactive catalytic chains exhibited homotropic and heterotropic effects characteristic of the native enzyme. Thus, it is not necessary for cooperativity that all catalytic chains in the complex be enzymically active.

Must there be at least one active chain in each trimeric catalytic subunit for the complex to exhibit cooperativity? The results with C\_nCp\_R show clearly that it is not necessary to have active chains in both subunits. This observation is particularly striking since the transiently stable complex, CR\_4 (16), composed of only one native C and three R subunits, exhibits neither homotropic nor heterotropic effects (18). Thus, direct interactions between native c and r chains are not sufficient to confer cooperativity (or inhibition) on the enzymically active chains in CR\_4. Linking the c and r chains in CR\_4 does affect the \( K_m \) of the catalytic chains (comparable to free C) (15), but the resulting structure permits the three active sites to function independently. When the other half of each R dimer is in turn “anchored” by being linked to a second C subunit, even an inactive one, the entire structure becomes constrained so that ligand binding to one chain affects active chains in the same subunit. According to this view, cooperativity is dependent on the architecture of the entire complex and the resulting quaternary constraint. There may be contributions to cooperativity from direct interactions between the two C trimers (even though one is inactive).†

Although interactions between the two c:r protomers (i.e., c:r:c) are important in providing a cooperative structure, it remains to be seen whether a hybrid containing only one active chain in each subunit, such as C\_nnp-C\_npp\_R, would exhibit cooperativity. In other words, is there communication of conformational changes across the regulatory subunit bridges? Reconstituted molecules like C\_nnp-C\_npp\_R and C\_nnp-C\_npp\_R would exist as a mixture of three isomeric species differing in the relative orientations of the native c chains in the two C subunits. Since the reconstitution is likely to produce a random collection of geometrical isomers, it is of special significance, as seen from C\_nCp\_R, that both c chains in the linked protomers, c:r:c, need not be active for cooperativity.

Both hybrids, C\_nCp\_R and C\_nnp-C\_npp\_R, show less cooperativity than does ATCase. The decrease in the Hill coefficient, \( n_H \), from 1.75 for C\_nCp\_R to lower values for the hybrids is expected, since there is a reduction in the number of active sites in the molecules. As yet we do not know whether the presence of pyridoxylated chains in the hybrids affects the transitions of the active chains from the putative constrained to relaxed conformations; hence a quantitative application of various models (4, 5) for allosteric enzymes is not warranted until additional data are obtained. Nonetheless, two observations cited in Table 1 are noteworthy. First, the conformational changes in the hybrids promoted by the addition of ligands are very similar to those found for ATCase. This is shown by the values of \( \Delta S/3 \) caused by carbamoylphosphate alone (about −0.5%) and the subsequent addition of the substrate analog succinate (about −2.6%). Second, the apparent \( K_m \) (for aspartate) increased from 7 mM for C\_nCp\_R to 12 mM and 11 mM for C\_nCp\_R and C\_nnp-C\_npp\_R, respectively. These increases relative to ATCase are a consequence of the reduction in the number of remaining active sites per molecule that can be converted from the low affinity form (constrained state) to a conformation with higher affinity (relaxed state) as a result of the allosteric transition accompanying the binding of ligands to the first site (or sites). The resemblance of the kinetic behavior of C\_nCp\_R and C\_nnp-C\_npp\_R suggests that the number rather than the distribution of active sites is of greater significance in the reduction of the cooperativity and the increase in apparent \( K_m \) in the hybrids. This hypothesis can be tested with studies on other hybrids like C\_nnp-C\_npp\_R and C\_nnp-C\_npp\_R.
ATCase hybrids containing native subunits and either catalytic or regulatory subunits that were extensively modified with dicarboxylic acid anhydrides have been found to be non-cooperative. The hybrid C\textsubscript{N}C\textsubscript{P}\textsubscript{R}[R] containing many THP-groups on the pyridoxylated catalytic subunit is no exception (see Table 1). Presumably the electrostatic repulsion in highly negatively charged ATCase-like molecules destabilizes the constrained state (G. M. Nagel & H. K. Schachman, in preparation) and causes a conformational change related to the allosteric transition either spontaneously or upon the binding of carbamoylphosphate; subsequent addition of aspartate or succinate would then have little effect, since the transition to the relaxed state was complete already, and the complexes, though enzymically active, exhibit no cooperativity. Indeed, the value of $\Delta G/\Delta$ for C\textsubscript{N}C\textsubscript{P}\textsubscript{R}[R] upon the addition of carbamoylphosphate is significantly larger than that for ATCase, and the subsequent change caused by succinate is smaller than usual\$.

These studies show the value of THPA for the reversible modification of amino groups in proteins and its use as a "chromatographic handle" in the preparation of hybrids of well-defined composition. The THP-amide bonds are sufficiently stable under the chromatographic conditions to permit separation of different species. Lowering the pH of the modified protein to about 5 permits the quantitative removal of the THP-groups to give the desired molecules devoid of extraneous charged groups. This anhydride should prove useful in many types of studies on proteins where reversible modification under mild conditions is essential.

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\$ Spectral measurements (M. N. Blackburn, unpublished observations) show that both C\textsubscript{P} and C\textsubscript{N}C\textsubscript{P}[R] bind carbamoylphosphate but not succinate. At the levels of carbamoylphosphate used in the experiments described above, all of the catalytic chains in the hybrids are probably saturated with this substrate. Hence, the observed cooperativity of the hybrids is that of the complexes containing carbamoylphosphate. Although binding of the second substrate, aspartate, to both C subunits is not required for cooperativity, it remains to be established whether carbamoylphosphate binding to both subunits is essential.