Ligand-promoted Strengthening of Interchain Bonding Domains in Catalytic Subunits of Aspartate Transcarbamoylase*

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Attempts to account for the allosteric behavior of enzymes in terms of a ligand-promoted conformational change from a constrained, low affinity state to a relaxed form of higher affinity for substrates require measurements of the changes in interchain interactions accompanying the allosteric transition. With aspartate transcarbamoylase of Escherichia coli this is a difficult task because of the multiplicity of interactions; these include six bonding domains among the catalytic chains in the two catalytic trimers, six bonding domains linking the catalytic and regulatory chains, and three bonds between the pairs of regulatory chains in the three regulatory dimers. Because of the difficulty in measuring independently the effect of active site ligands on the various types of interchain bonding domains, we have utilized isolated catalytic subunits to determine whether the same ligands which promote the allosteric transition of the intact enzyme cause a change in the strength of the bonds linking the catalytic chains in the nonallosteric catalytic trimers. Native catalytic subunits are extremely stable and exhibit little tendency to dissociate into single chains in neutral solutions. Hence hybridization experiments with native and succinylated subunits were used as a sensitive technique to measure the rate of dissociation of the catalytic trimers. The half-time for dissociation at 0 °C was about 75 h and the rate was even slower at 25 °C. Moreover, the substrate carbamoyl phosphate and the bisubstrate analog, N-(phosphonacetyl)-L-aspartate, caused a marked strengthening of the bonds between catalytic chains in the subunits. These results on the bonds between catalytic chains differ strikingly from those for the bonds between catalytic and regulatory chains. Although the conformation of free catalytic subunits is doubtless different from that of subunits within the intact enzyme, the results showing the ligand-promoted strengthening of the bonds between catalytic chains are likely to be important in accounting for the cooperativity of aspartate transcarbamoylase in terms of interchain interactions.

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The homotropic and heterotropic effects exhibited by some allosteric proteins can be attributed to ligand-promoted conformational changes whereby the proteins are converted from a constrained or low affinity state to a relaxed form having a higher affinity for substrates (1). Because these conformational changes are mediated by alterations in subunit interactions (2), it is important to determine the strengths of the interchain bonding domains and the changes in interaction energies caused by ligands. Only rarely has this been achieved (3-6), and in the case of the regulatory enzyme, aspartate transcarbamoylase (carbamoylphosphate:L-aspartate carboxytransferase, EC 2.1.3.2) from Escherichia coli (7), the evaluation of changes in subunit interactions is particularly difficult because of the large number and different types of noncovalent interchain bonding domains (8). In this paper we demonstrate that the same active site ligand which promotes a weakening in the "bonds" between the catalytic and regulatory chains (9) causes a marked strengthening in the bonding domains between the polypeptide chains in isolated catalytic subunits (10). ATCase is composed of two catalytic trimers (C) and three regulatory dimers (R) with each catalytic polypeptide chain (c) in one C subunit linked noncovalently to a c chain in the other C subunit via an R subunit (11-15). In this structure, designated C2R3 or C6R6, there are six c:c bonding domains within the two C trimers, three r:r bonding domains linking the r chains within the R dimers, and six c:r bonding domains connecting the c and r chains (8, 15). Although there is a large aqueous cavity between the two C subunits in the center of the molecule (16), recent evidence from x-ray diffraction studies indicates that some atoms in each of the C subunits are in direct contact (17). Because of the multiplicity of interchain interactions and their intrinsic strengths, ATCase, unlike many other oligomeric enzymes, exhibits little tendency to dissociate into subunits even at concentrations as low as 10⁻⁵ M in buffers at neutral pH (18). Moreover, very little exchange of either C or R subunits was detected when radioactive subunits were incubated with native or reconstituted ATCase (9, 19). Hence, experiments on native ATCase, with one ex-
exception (20), have not provided quantitative information regarding changes in interchain interactions when the enzyme is converted from the taut (T) state to the relaxed (R) conformation.

An estimate of the ligand-promoted change in the c:r bond strength has been determined with ATCase-like molecules lacking one R subunit (9). This allosteric, R-deficient species, C2R2 (21–24), which contains only four c:r bonds, is less stable than the native enzyme and, under certain conditions, disproportionate to yield ATCase and free C subunits (9, 25). The rate-limiting reaction in this disproportionation process has been shown to involve the rupture of two c:r bonds (25) and the addition of the bisubstrate ligand, PALA, which binds portionately to yield ATCase and free C subunits. Therefore, studies were conducted on isolated, nonallosteric, C subunits in an effort to determine whether C,R deficient species, have been useful for evaluating possible changes in the strength of interchain interactions are affected by the same active site ligand, PALA, which promoted the T→R transition in intact ATCase and C2R2. Although C subunits are very stable in neutral solutions and show no evident dissociation in sedimentation velocity experiments at 3 μg/ml (27), hybrids could be detected when native (Cn) and succinylated (Cn) subunits were incubated together for prolonged periods of time. Thus, the formation of hybrids was used as a measure of the dissociation of the trimers which in turn was an indication of the strength of the c:c bonding domains.

MATeRiALS AND METHODS

ATCase was prepared according to the procedure of Gerhart and Holoubek (28) and the subunits produced by treating ATCase with neohydrin were separated by chromatography on DEAE-cellulose (29). C subunits were succinylated (13) at a 2.5 molar ratio of succinimide to yield a derivative, Cn, containing about four succinyl groups per chain. 125I-labeled C subunits were prepared by the procedure of Syvanen et al. (30) and the specific activity of the modified protein was about 5 × 10⁶ cpm/μg.

Succinic anhydride was purchased from Eastman, neohydrin from K & K Laboratories, and carrier-free 125I as the sodium salt in NaOH was obtained from Amersham Corp. PALA was kindly provided by Dr. G. R. Stark of Stanford University.

Hybridization was performed by incubating Cn (Cn) and Cs (Cn) in 40 mM Tris-HCl at pH 8.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA for various lengths of time. Both protein concentration and temperature were varied and the indicated pH values were those measured at the temperature of the experiment (see "Results"). The four members in the hybrid set, Cnn, Cns, Csn, and Css, were separated electrophoretically in 7.5% polyacrylamide gels (6 cm with a 2-cm stacking gel). The discontinuous Tris-barbital system described by Gabriel (31) was employed and gels were stained with Coomassie brilliant blue G-250 (32). In experiments with radioactively labeled proteins, the gels were sliced and the radioactivity measurements on the fractions were made with a Nuclear-Chicago γ-counter.

RESULTS

c:c Bonding Domains Are Strong as Indicated by Slow Rate of Hybrid Formation between Cnn and Cnn.—Prolonged incubation of Cnn subunits with Cnn subunits is required for the formation of significant amounts of the hybrids, Cnn and Cnn. The rate of hybridization depends on the pH, ionic strength, and specific anions and, as yet, these effects have not been investigated systematically. As seen in Fig. 1, the formation of significant amounts of Cnn and Cnn at 0 °C and pH 8.0 required 94 h. In this experiment the initial concentrations of Cnn and Cnn were the same and the hybrids were produced in approximately equal amounts.

Quantitative measurements of the rate of dissociation of Cnn trimers were performed by incubating mixtures of 125I-labeled Cnn subunits and unlabeled Cnn trimers in a 1:10 ratio.

FIG. 1. Hybrid formation upon incubation of a mixture of Cnn and Cnn. The mixture containing Cnn and Cnn, each at 1 mg/ml, was incubated at 0 °C in 40 mM Tris-HCl at pH 8.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. At designated times aliquots were removed and analyzed by electrophoresis in polyacrylamide gels as described under "Materials and Methods." A, analysis immediately after mixing Cnn and Cnn; B, electrophoresis after 94 h of incubation.

FIG. 2. Effect of temperature on the strength of c:c and c:r bonding domains. a, hybrid formation in mixture of Cnn and Cnn. Mixtures containing 125I-labeled Cnn at 0.3 mg/ml and unlabeled Cnn at 3 mg/ml in 40 mM Tris-HCl at pH 7.3 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA were incubated at 0 and 25 °C. The ionic strength of the solution at 25 °C was adjusted with KCl to that of the solution at 0 °C. Aliquots were withdrawn at designated times, the different species were separated electrophoretically, and the fraction of radioactivity migrating with the mobility of Cnn was determined. Results at 0 °C (A) and at 25 °C (B) are expressed as the percentage of Cnn remaining as a function of time, b, disproportionation of Cnn to form ATCase and free C subunits. Results of Subramani et al. (9) are shown as the percentage of Cnn remaining at 0 °C (A) and at 25 °C (B) as a function of time.

Any native monomers (Cn) produced by dissociation of the native subunits preferentially form complexes with succinylated monomers (Cn) to yield Cnn hybrids. Similarly, Cn dimers which are only marginally stable (33) would combine rapidly with Cn monomers to form Cnn hybrids. Thus, the amount of Cnn trimers remaining as a function of time provides a direct measure of the rate of rupture of c:c bonds in the native subunits. Fig. 2a shows that the half-time for dissociation of the Cnn subunits was about 75 h at 0 °C. The rate of dissociation at 25 °C was much slower; so little dissociation occurred in 150 h that the half-time could not be determined accurately.

For comparative purposes we have included in Fig. 2b the results of Subramani et al. (9) on the disproportionation of Cn to form Cn and free C subunits. As seen in Fig. 2b, the

2 The effect of temperature on the rate of hybridization varied with different buffers. When the experiment was performed with Tris-HCl (pH 7.5) or potassium phosphate (pH 7.0) as the buffer, hybridization occurred significantly more rapidly at 0 °C than at 25 °C as seen in Fig. 2a. However, when 4-(2-hydroxyethyl)-2-piperazinepropanesulfonic acid was used as the buffer, the rate of hybridization was virtually the same at the two temperatures.
Domains—Fig. 2. 

Fig. 3. Effect of substrate, carbamoyl phosphate, on the extent of hybrid formation in mixture of C<sub>ss</sub> and C<sub>ns</sub>. Experiments were performed as in the legend to Fig. 1 with mixtures containing equal amounts of C<sub>ss</sub> and C<sub>ns</sub> at 1 mg/ml. The solutions were incubated at 0 °C for 100 h in 40 mM Tris-HCl and 0.1 mM 2-mercaptoethanol and 0.2 mM EDTA. The electrophoresis pattern in a is the control containing no ligands and that in b shows the effect of carbamoyl phosphate at 4 mM.

rate of disappearance of C<sub>ss</sub>R<sub>ss</sub>, which depends on the rupture of cr bonds (25), is greater at 25 °C than at 0 °C. Thus, the cr bonds in C<sub>ss</sub>R<sub>ss</sub> are weaker at 25 °C than at 0 °C, whereas the cr bonds in isolated C subunits are stronger at 25 °C than at 0 °C.

Active Site Ligands Increase the Strength of cr Bonding Domains—Fig. 3 shows the effect of the substrate, carbamoyl phosphate, on the extent of hybrid formation when C<sub>ss</sub> and C<sub>ns</sub> were incubated at 0 °C for 100 h at pH 8.0. Significant amounts of both C<sub>ss</sub> and C<sub>ns</sub> were formed in the control experiment containing no ligand. However, very little of either hybrid could be detected when the incubation mixture of C<sub>ss</sub> and C<sub>ns</sub> contained the substrate, carbamoyl phosphate, at 4 mM. Analogous hybridization experiments with mixtures of the two proteins in the presence of the bisubstrate analog, PALA, also showed virtually no hybrid formation during the same time interval. In contrast, succinate, which is an analog of the substrate, aspartate, had virtually no effect on the rate of hybrid formation; the electrophoretic pattern was virtually identical to that obtained when no ligands were present. When both carbamoyl phosphate and succinate were present together, the pattern was similar to that observed for the incubation mixtures containing either carbamoyl phosphate alone or PALA.

The hybridization experiments demonstrate clearly that ligands which bind at the active sites of the enzyme cause a marked decrease in the rate of dissociation of the subunits, which can be attributed to a strengthening of the cr bonding domains.

Kinetic measurements of the rate of hybrid formation were made with mixtures of carrier-free labeled C<sub>ss</sub> and unlabeled C<sub>ns</sub> in both the absence and presence of PALA. The dramatic effect of PALA in decreasing the rate of disappearance of C<sub>ss</sub> is shown in Fig. 4a. This experiment was performed with the same preparations of carrier-free labeled C<sub>ss</sub> and unlabeled C<sub>ns</sub> as those used for Fig. 2. However, the ratio of the two proteins in Fig. 4a was 1:1 as contrasted to 1:10 for the experiment in Fig. 2a. Hence the slower observed disappearance of C<sub>ss</sub> in Fig. 4a in the absence of any ligand as compared to the kinetics in Fig. 2a can be attributed to the accumulation in the former of significant amounts of C<sub>ss</sub> and C<sub>ns</sub> which in turn tend to disproportionate to form some C<sub>ss</sub> and C<sub>ns</sub>.

Fig. 4b shows the effect of PALA on the rate of disproportionation of C<sub>ss</sub>R<sub>ss</sub> (9). The cr bonds are weakened to a very large extent due to the binding of PALA at the active sites (9). Thus, as was observed for the temperature dependence, the strengths of the cr bonds and the cr bonds are affected in opposite directions by the bisubstrate analog.

**DISCUSSION**

Although it has been demonstrated that ATCase undergoes a gross conformational change upon the addition of active site ligands (18, 34-38) and that the alteration in quaternary structure is linked to changes in the tertiary structures of both the c and r chains (39-42), our knowledge of the structural differences between the T- and R-states is still meager. Hence it is not yet possible to correlate thermodynamic parameters for the T→R transition evaluated from enzyme kinetics (18) with structural differences between the two conformations. An intermediate goal in this overall effort to describe the transition in molecular detail is the evaluation of the changes in the interchain interactions. Even this limited objective is difficult to achieve because of the multiplicity of bonding domains and their intrinsic strengths.

Each r chain in ATCase is linked noncovalently to one other r chain and to one c chain (8). Similarly each c chain is linked to two other c chains in the same C subunit and to one r chain (8). In addition, there is some slight contact between each c chain in one subunit and another c chain in the other subunit (17). Hence, it is not surprising that no significant exchange of polypeptide chains has yet been demonstrated when ATCase is incubated with free C or R subunits in neutral solutions. Because of this stability of ATCase, the less stable, R-deficient species, C<sub>ss</sub>R<sub>ss</sub>, has been utilized for preliminary assessments of the weakening of the cr bonding domains accompanying the T→R transition (9). These studies indicated that each cr bond is weakened by about 1.7 kcal/mol in the conversion of the molecules from the T→R transition (9). If this value were to be applied to ATCase, the total decrease in the cr bond strengths would be 10 kcal/mol for the T→R transition. In contrast, the free energy difference evaluated from enzyme kinetics is only −3.3 kcal/mol for the

It should be noted that the enzyme kinetics was measured in solutions of much larger ionic strength than those which yielded the value, 1.7 kcal/mol, for the weakening of a cr bond caused by the...
Strength of Interchain Bonds in Catalytic Trimers

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ligand-promoted conformational change (18). Thus, it is important to determine whether there are any compensating changes in the cc bonding domains upon the binding of active site ligands. Since the effects of these ligands on the cc bonding domains cannot be determined directly with either ATCase or Cr, we have used isolated C subunits for this purpose.

As seen in Figs. 1 and 2, dissociation of the trimers is very slow and the cc bonds are stronger at 25 °C than at 0 °C. Moreover, these bonding domains are strengthened markedly by the addition of ligands which bind at the active sites (Fig. 3). These results on the cc bonds are in marked contrast to those for the cr bonding domains in Cr (9) which are weakened at 25 °C (relative to 0 °C) and in the presence of the bisubstrate ligand, PALA, which promotes the T→R conversion (Figs. 2b and 4b).

Quantitative estimates are not yet available for the magnitude of the strengthening of the cc bonding domains upon the binding of active site ligands. The extent of hybridization under the conditions used here is so slight that accurate rates of dissociation of the trimers both in the absence and presence of substrate analogs cannot be determined. Although the rate can be enhanced markedly by the presence of specific anions so that the effect of ligands in strengthening the cc bonds could be measured quantitatively, it is not clear that the data obtained from such experiments could be applied to an analysis of the ligand-promoted changes in interchain interactions in intact ATCase. How much change in the cc bonding domains occurs when the subunits are incorporated into ATCase is not known. It seems reasonable, nonetheless, to assume that some strengthening of the cc bonds observed with isolated C subunits upon the addition of active site ligands may also occur in the intact enzyme.

Two independent sets of observations suggest that the cc bonding domains are implicated in an important way in mediating the cooperativity exhibited by ATCase. First, the cc chains which are not bonded to cr chains in Cr exhibit the kinetic properties of ATCase and not of free C subunits (22). Despite the absence of cr bonds for those cc chains, there is sufficient communication (and constraint) through the cc bonding domains that those cc chains have a Vmax characteristic of the intact enzyme which is significantly greater than Vmax for chains in isolated C subunits (22). Second, in mutant forms of ATCase which exhibit markedly different allosteric properties the magnitude of the cooperativity is related to the increasing strengths of the cc bonds.5

The observed strengthening of the cc bonds upon the addition of active site ligands to isolated C subunits is relevant to earlier findings on the effect of these ligands on the physical properties of the protein. Succinate, for example, has no detectable effect on the strength of the cc bonds and on the sedimentation coefficient of the protein (45), and causes only a slight perturbation of the near ultraviolet absorption spectrum (46). These results are consistent with the data from equilibrium dialysis experiments which show that the binding of succinate to C subunits is very weak (47). Carbamoyl phosphate, however, causes a marked increase in the strength of the cc bonds (Fig. 4) and a slight increase in the sedimentation coefficient of the C subunits (35), and a significant perturbation of tyrosine and tryptophan residues leading to a small difference spectrum with sharp peaks at 281.2 and 288.6 nm and a low, broad peak at 300 nm (46).

When both carbamoyl phosphate and succinate are added together to the C subunits, the cc bonds are strengthened, and there is a significant increase in the sedimentation coefficient of the protein (35, 45) as well as a marked perturbation of the near ultraviolet absorption spectrum which is considerably greater than that calculated from the sum of the spectra caused by the individual ligands separately (46). Moreover, the binding of succinate is enhanced considerably when carbamoyl phosphate is also present (46, 47). The sedimentation and spectral studies indicate that the addition of the two ligands together causes a significant conformational change in the C subunits which has been interpreted as a contraction or compression of the protein (35, 45, 46). This combined effect of the two ligands is observed as well when the bisubstrate ligand, PALA, is added alone. The cc bonds are strengthened (46), the sedimentation is increased (50), and the spectrum of both tyrosine and tryptophan residues is perturbed (26). Moreover, differential scanning calorimetric measurements have shown that the binding of PALA causes a significant increase in the temperature at which C subunits are denatured (48).

The strengthening of the cc bonds by ligands which bind at the active sites is particularly interesting in the light of the suggestion from x-ray diffraction studies (17) that the active sites in ATCase are shared between adjacent cc chains within the C trimers. This proposed joint participation of pairs of chains in the formation of an active site has been invoked recently in speculations aimed at accounting for the effects of various ligands on the rate of hydrogen exchange in tritiated C subunits (49). In these studies the much larger effect of PALA, compared to carbamoyl phosphate, in decreasing the rate of exchange was interpreted as the result of the bisubstrate ligand acting as a bridge between the chains. Carbamoyl phosphate was assumed to be unable to serve effectively in this way. Our findings show that carbamoyl phosphate, like PALA, causes a marked strengthening of the cc bonds.5 It should be noted, however, that as yet no quantitative data of their relative effectiveness are available. Thus far, definitive evidence has not been presented which permits the identification of the amino acids that contribute to the binding of substrates or their analogs. If PALA is bound through contacts with two chains, then strengthening of the cc bonds would be expected. Those contacts would have to account as well for the significant increase in bond strength caused by the much smaller ligand, carbamoyl phosphate. When the complete amino acid sequence of the cc chains is determined and a more refined structure of the C subunits is available (17, 51), it should be possible to interpret the findings presented here.

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5 In the experiments involving prolonged incubation of Cmax, and Crmax in the presence of carbamoyl phosphate there may be some carbamylation of the protein due to cyanate produced by the breakdown of carbamoyl phosphate (50). Since succinylation of Cmax (under conditions which inactivate the protein and cause significant modification of (lysine residues) weakens cc bonds in a manner unlikely that nonspecific, slight carbamylation of the protein could cause a strengthening of the cc bonds. Our conclusion that active-site ligands cause a strengthening of the cc bonds is reinforced, of course, by the results with PALA which does not cause covalent modification of the protein.

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