Ligand-promoted weakening of intersubunit bonding domains in aspartate transcarbamoylase

(protein-protein interactions/conformational changes/allosteric enzymes/kinetics of assembly)

SURESH SUBRAMANIA^a, MARK A. BOTHWELL^b, IAN GIBBONS^c, YING R. YANG, AND H. K. SCHACHMAN

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

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ABSTRACT The cooperativity and feedback inhibition exhibited by the regulatory enzyme, aspartate transcarbamoylase (carbamoylphosphate: L-aspartate carbamoyltransferase; EC 2.1.3.2), from *Escherichia coli* are generally attributed to ligand-promoted conformational changes involving alterations in the subunit interactions. However, no quantitative estimates have been made of the effect of ligands on the strength of the intersubunit bonding domains. The native enzyme, composed of two catalytic trimers "crosslinked" by three regulatory dimers, shows little tendency to dissociate in neutral buffers at room temperature. In addition, very little exchange was observed in 2 hr between subunits within the intact enzyme and free subunits. Although exchange was enhanced in solutions of low ionic strength containing the bisubstrate analog, *N*-(phosphonacetyl)-L-aspartate, the rates of exchange were too small to permit reliable estimates of the weakening of the bonding domains caused by the ligand. Studies were conducted, therefore, on a less stable oligomeric complex which resembles the native enzyme in structure and allosteric behavior but lacks one regulatory subunit. These molecules, containing only four bonding domains between the catalytic and regulatory polypeptide chains (compared to six in the native enzyme), disproportionate to form the more stable native enzyme and free catalytic subunits. An electrophoretic technique is described for measuring the rate of disproportionation which is controlled by the rupture of the intersubunit bonding domains. This rate is enhanced about 300-fold upon the addition of the active-site ligand. Hence the ligand-promoted allosteric conversion of the enzyme-like complex from the constrained to the relaxed conformation involves a substantial weakening of the intersubunit interactions corresponding to about 1.7 kcal/mole (7.1 kJ/mole) per bonding domain between a catalytic and a regulatory chain.

It is generally recognized that the homotropic and heterotropic effects exhibited by allosteric enzymes are attributable 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, 2). Since these conformational changes are mediated by alterations in the subunit interactions, it is important to determine the strengths of the intersubunit bonding domains and their perturbation by ligands (3). Despite the evident need for such data, quantitative estimates of ligand effects are available for only a few proteins. Of these, hemoglobin has been studied most extensively. The dissociation constants for the tetramer-dimer equilibria have been evaluated for hemoglobin in both the oxy and deoxy states (4-9), and the changes in the intersubunit contact energies have been related to the functional properties of the protein (10, 11). In this paper we present an approach aimed at providing comparable information for the regulatory enzyme, aspartate transcarbamoylase (ATCase; carbamoyl-

phosphate:L-aspartate carbamoyltransferase; EC 2.1.3.2) from *Escherichia coli* (12).

The native enzyme is composed of two catalytic trimers (C) and three regulatory dimers (R) with each catalytic polypeptide chain (c) in one C subunit linked to a c chain in the other C subunit via an R subunit (13-17). In this structure, designated C₂R₃ or c₆r₆, there are six c-c bonding domains within the two C trimers, three r-r domains linking the regulatory polypeptide chains, r, within the three R dimers, and six c-r domains connecting the c and r chains (18).^e Because of this multiplicity of bonding domains and their intrinsic strengths, ATCase, unlike many other oligomeric enzymes, exhibits little tendency to dissociate into subunits in buffers at neutral pH. No dissociation at 10⁻⁸ M was observed in sedimentation velocity experiments (19). Even at 10⁻¹⁰ M, ATCase exhibited the homotropic and heterotropic effects characteristic of the intact enzyme (20).

Since the dissociation of ATCase into free subunits could not be measured directly, we initiated studies of subunit exchange as an alternative, sensitive approach for estimating the strengths of the intersubunit bonding domains. Very little exchange of either C or R subunits occurred even in the presence of the bisubstrate analog, *N*-(phosphonacetyl)-L-aspartate (PALA), which promotes the conformational transition of ATCase to the relaxed state (20, 21). Studies were conducted, therefore, on the effect of this active-site ligand on the bonding domains in ATCase-like molecules lacking one R subunit (22-25). This R-deficient species, designated C₂R₂, which exhibits both cooperativity and inhibition by CTP (22, 23), is an intermediate in both the *in vitro* assembly of ATCase from subunits and the dissociation of the enzyme (24, 26). Since there are only four c-r bonding domains in C₂R₂ as compared to six in ATCase,

Abbreviations: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; C₂R₃, subunit description of ATCase in terms of two C and three R subunits; C₂R₂, an ATCase-like molecule containing two C and two R subunits; c, catalytic polypeptide chain; r, regulatory polypeptide chain; PALA, *N*-(phosphonacetyl)-L-aspartate; *C, ¹²⁵I-labeled C subunit; *R, ¹²⁵I-labeled R subunit; *ATCase, ATCase labeled with ¹²⁵I in both C and R subunits.

^a Submitted in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry.

^b Present address: Department of Neurobiology, Stanford University Medical Center, Stanford, CA 94305.

^c Present address: Department of Biochemistry, University of Bristol, Bristol BS8 1TD, England.

^d To whom requests for reprints should be addressed.

^e This is the simplest pattern of bonding domains compatible with available structural information that the C subunits are not in direct contact and that the R subunits are on the outside of the molecule (17). Whether additional domains must be considered will have to await the results of further x-ray diffraction studies. It is possible, for example, that a single r chain makes contact with two c chains in each C subunit; in that case there would be two different classes of c-r bonding domains with six of each type. Other interactions can be visualized as well.

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C_2R_2 would be expected to be less stable than ATCase. Indeed, under certain conditions, C_2R_2 disproportionates to form ATCase and free C subunits (23, 24, 26). This process, $3 C_2R_2 \rightarrow 2 C_2R_3 + 2 C$, was followed by electrophoresis and the data were analyzed to give estimates of the rate of rupture of the c-r bonding domains and the effect of the active-site ligand on the strength of the subunit interactions.

MATERIALS AND METHODS

Chemicals. Carrier-free ^{125}I was obtained as the sodium salt, in NaOH solution at pH 8.0–10.0, from Schwarz Mann, Inc. PALA was a gift from G. R. Stark (21). Carbamoyl phosphate, as the dilithium salt, was crystallized from ethanol and fresh solutions were made for each experiment. Solutions of carbamoyl phosphate and succinate were adjusted to pH 7.5 in H_2O . Neohydrin [1-(3-chloromercuri-2-methoxypropyl)urea] was purchased from K & K Laboratories, Inc. and purified from an alkaline solution by the addition of HCl. Coomassie Brilliant Blue was obtained from the Serva Chemical Co. and methyl-*p*-hydroxybenzimidate-HCl from Pierce Chemical Co.

Preparation of ATCase, C_2R_2 , and C and R Subunits. ATCase was prepared according to Gerhart and Holoubek (27). The subunits of ATCase, C and R, were prepared by the complete dissociation of ATCase with neohydrin followed by chromatography on DEAE-cellulose (28). C_2R_2 was prepared by partial dissociation of ATCase with neohydrin (24). After chromatography, it was stored in 50 mM Tris-HCl/8 mM 2-mercaptoethanol at pH 7.5. Protein concentrations were determined spectrophotometrically at 280 nm. The extinction coefficients for C, R, C_2R_2 , and C_2R_3 were 0.72, 0.31, 0.62, and $0.59 \text{ cm}^2\text{mg}^{-1}$, respectively.

Preparation of ^{125}I -Labeled Proteins. The procedure of Syvanen *et al.* (29) was used for labeling the C subunit, which was stored in 40 mM Tris-HCl/2 mM 2-mercaptoethanol at pH 7.5 and used within 4–5 days. The specific activity of labeled C (*C) was $10^5 \text{ cpm}/\mu\text{g}$ and the protein was greater than 95% reconstitutable with an excess of R subunits. ^{125}I -Labeled R (*R) was prepared by reacting R with ^{125}I -labeled methyl-*p*-hydroxybenzimidate-HCl (24, 30). Intact ATCase labeled randomly in both the C and R subunits (*ATCase) with 3,5-diiodo-methyl-*p*-hydroxybenzimidate was kindly supplied by M. M. Wu and J. C. Gerhart. The ^{125}I was distributed about equally on the *C and *R subunits. Radioactivity was measured with a Nuclear Chicago γ -counter.

Electrophoresis. The Tris-glycine system of Jovin *et al.* (31) was used for electrophoresis of samples in polyacrylamide gels (7% with 2 cm of 2.5% stacking gel). Coomassie Brilliant Blue G-250 was used for staining the gels (32), which were scanned on a Joyce-Loebl microdensitometer fitted with a red filter. The percentages of C_2R_3 , C_2R_2 , and c were evaluated from the tracings by measuring the weight of the peak corresponding to a specific component relative to the combined weight for the three species. In some experiments independent analyses were made by scanning the gels on a Gilford spectrophotometer and the areas of the tracings were measured with a planimeter. Identical results were obtained by the two procedures. The areas corresponding to the individual components varied linearly with the amount of protein added to the gel up to a level of about 3 μg of any specific species. For the exchange experiments the gels were sliced after staining and the radioactivity of the various species was measured as indicated above.

Disproportionation Experiments. The conversion of C_2R_2 into C_2R_3 and C was studied both in the presence and absence of PALA at protein concentrations of about 0.06 mg/ml. For those disproportionation experiments involving PALA (suffi-

Table 1. Lack of exchange of free *C with subunits in ATCase†

Time of reaction, min	No ligands		PALA	
	Low ionic strength‡	High ionic strength‡	Low ionic strength§	High ionic strength¶
0	1.3	2.0	2.1	2.4
5	1.3	2.0	1.7	—
15	1.7	—	1.9	—
30	1.3	—	1.9	1.5
60	1.5	2.0	2.6	1.6
120	3.7	2.0	2.2	3.8

† Results are expressed as % radioactivity in ATCase. Exchange reaction was at 25° in buffers containing 2 mM 2-mercaptoethanol at pH 7.5, with low ionic strength corresponding to 4 mM Tris-HCl and high ionic strength to 50 mM Tris-HCl. For the experiments at low ionic strength, aliquots (100 μl) were removed at the designated times and the exchange was stopped by the addition of 10 μl of 500 mM Tris-HCl containing 2 mM 2-mercaptoethanol at pH 7.5. No stopping solution was used for the experiments at high ionic strength. Tracking dye (10 μl) was added to each aliquot, and 100 μl was loaded on the polyacrylamide gels for electrophoresis.

‡ *C (0.3 μM) was added to a mixture of ATCase and C_2R_2 , each at 0.1 μM .

§ Solution contained 0.2 μM *C, 0.14 μM ATCase, and 55 μM PALA. Purified ATCase was obtained by reacting C_2R_2 with an excess of R subunits (23) followed by chromatography on a Sephadex G-200 column.

¶ *C (0.88 μM) was added to a mixture of ATCase and C_2R_2 , each at 0.1 μM . PALA concentration was 33 μM .

cient to saturate the six catalytic sites), the zero time value was determined by removing an aliquot 15 sec prior to the addition of the ligand. This procedure was adopted since the rate of disproportionation at pH 7.5 in the absence of PALA was very low and because in the few minutes after the addition of PALA there was a marked conversion of C_2R_2 into C_2R_3 and C. Kinetics were measured by removing aliquots from the reaction mixture, adding concentrated buffer to give a final concentration of 50 mM Tris-HCl/2 mM 2-mercaptoethanol at pH 7.5, and analyzing the sample by electrophoresis. As shown below, C_2R_2 is stable in this buffer (even in the presence of PALA), and thus this method of stopping the disproportionation process is effective. All samples corresponding to different times in a given kinetic experiment were analyzed together, and the various gels were stained and scanned in an identical manner in order to minimize experimental errors. Unless otherwise noted, disproportionation experiments were performed at $25 \pm 0.5^\circ$.

RESULTS

Stability of ATCase. Studies were conducted in order to determine whether free C or R subunits labeled with ^{125}I , i.e., *C or *R, could be incorporated into ATCase by exchanging with the subunits within the intact enzyme. Representative data from experiments involving mixtures of ATCase and *C are summarized in Table 1. There was practically no incorporation of *C into ATCase in a 2-hr period in solutions of low (4 mM) or high (50 mM) ionic strength. A small amount of radioactivity in the gels at the position corresponding to ATCase represents background ^{125}I that apparently was not covalently bound to *C. There was little or no time-dependent increase in the radioactivity associated with ATCase, indicating the absence of exchange of C subunits under these experimental conditions. Similarly, as seen in Table 1, there was no exchange in solutions containing the bisubstrate analog, PALA.

Analogous experiments with ATCase and *R in the presence of PALA showed no exchange of R subunits (Table 2). The background of radioactivity in these studies was higher than

Table 2. Lack of exchange of free *R with subunits in ATCase in the presence of PALA

Time of reaction, min	Amount of radioactivity in ATCase, %	
	Low ionic strength	High ionic strength
1	2.9	3.0
3	3.0	5.0
5	3.0	4.8
10	2.4	2.3
20	2.7	2.8
60	3.3	2.1
90	3.5	2.6
120	3.9	2.6
140	3.5	2.3

Exchange reactions were performed at 25° with *R at 2.6 μ M, ATCase at 0.17 μ M, and PALA at 55 μ M. The low and high ionic strength buffers were the same as those described in Table 1 except that 0.1 mM Zn²⁺ was also present in order to stabilize the R dimers (17). The experimental procedures were the same as those for Table 1. About 85% of the *R subunits were converted into ATCase when mixed with excess C₂R₂. As an additional test of the competence of *R in the reaction mixtures, aliquots were removed and neohydrin was added to a concentration of 6 mM so as to cause the dissociation of the ATCase into C and R subunits (24). After 30 min, 2-mercaptoethanol was added to a concentration of 200 mM. This procedure leads to the reconstitution of ATCase. At least 30% of the radioactivity of *R was converted into ATCase in this process.

those shown in Table 1, but the amount of ¹²⁵I in the position on the gels corresponding to ATCase did not increase significantly in 2 hr.

Since the labeling of the free subunits might have rendered them less "competent" for incorporation into ATCase, we conducted similar experiments with *ATCase and unlabeled subunits. The randomly labeled *ATCase (0.185 μ M) was mixed for various times with unlabeled R subunits (2.1 μ M) in 50 mM Tris-HCl/2 mM 2-mercaptoethanol/0.2 mM Zn²⁺ at pH 7.5 in the presence of PALA (47 μ M), and the components were separated by electrophoresis. The amount of radioactivity associated with the free R subunits was 1.9, 1.8, 1.8, 1.9, 1.9, and 1.8% at 0, 5, 10, 40, 120, and 150 min, respectively. Thus these data are in agreement with the results of Table 2; *R subunits within *ATCase do not exchange with free R subunits in the high ionic strength buffer containing PALA. When PALA was absent, the amount of radioactivity associated with the free R subunits was only 1.5% after 160 min.

In a similar experiment with *ATCase in the low ionic strength buffer containing PALA and 0.1 mM Zn²⁺ to stabilize the R dimers (17), there was a significant but slow exchange of R subunits. After incubation of *ATCase with free R subunits for 0, 5, 20, 60, 90, and 120 min, the amounts of radioactivity associated with the free R subunits were 1.6, 2.2, 3.4, 4.4, 4.7, and 5.2%, respectively. This slight exchange should be contrasted with the absence of exchange in the analogous experiment without PALA; at corresponding times the amounts of radioactivity found with the free R subunits were 0.7, 1.0, 0.9, 0.8, 1.0, and 1.0%. After 18 hr the amount was only 1.0%.

Disproportionation of C₂R₂. In earlier studies on the structure and properties of C₂R₂ we observed that storage of C₂R₂ for several weeks in 50 mM Tris-HCl containing 2 mM 2-mercaptoethanol at pH 7.5 led to its partial conversion into a mixture of ATCase and free C subunits (23). Moreover, the disproportionation of C₂R₂ was more rapid in low ionic strength buffers (5 mM Tris-HCl) and was markedly accelerated upon the addition of PALA (24).

The time dependence and the effect of ligands on the dis-

FIG. 1. Electrophoretic analysis of the disproportionation of C₂R₂ into ATCase (C₂R₃) and free C subunits. Experiments were on 7% polyacrylamide gels at a protein concentration of about 0.06 mg/ml. The buffer for the disproportionation at 25° was 4 mM Tris-HCl containing 2 mM 2-mercaptoethanol at pH 7.5. (A and B) Solutions containing no PALA; 10 (A) and 70 (B) min of incubation of the samples prior to loading the gels. (C, D, and E) Samples containing a saturating amount of PALA (50 μ M); 10 (C), 40 (D), and 70 (E) min of reaction.

proportionation of C₂R₂ are shown qualitatively by the electrophoretic patterns in Fig. 1. For these experiments the buffer contained 4 mM Tris-HCl and 2 mM 2-mercaptoethanol at pH 7.5. The preparation contained initially about 90% C₂R₂, 5% ATCase, and 5% free C subunits (Fig. 1A). Storage of this solution for 70 min followed by electrophoresis yielded the pattern in Fig. 1B, which showed a very slight increase in the amount of ATCase and free C subunits. Hence disproportionation of C₂R₂ in the absence of ligands, even in the low ionic strength buffer, was slow. In contrast, C₂R₂ was converted into ATCase and free C subunits very rapidly when PALA was present. The pattern in Fig. 1C was obtained with a preparation of C₂R₂ after 10 min of exposure to a saturating amount of PALA; those in Fig. 1D and E illustrate the disproportionation after 40 min and 70 min, respectively. Whereas most of the C₂R₂ was still intact after 70 min in the absence of ligands (Fig. 1B), the bulk of the C₂R₂ had disproportionated in the same time period when PALA was present (Fig. 1E).

Stopping the Disproportionation of C₂R₂ by Increasing the Ionic Strength. For a quantitative kinetic analysis of the disproportionation process it was necessary to develop a method of stopping the disproportionation prior to the analysis of the products by electrophoresis. In the time periods used in this work no disproportionation of C₂R₂ was detected in buffers of higher ionic strength; i.e., 50 mM Tris-HCl/2 mM 2-mercaptoethanol at pH 7.5. Moreover, C₂R₂ exhibited both homotropic and heterotropic effects at a protein concentration of only 10⁻⁹ M (23). Hence the complex remained intact even in the presence of substrates in 50 mM imidazole buffer containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA at pH 7.0. Further evidence for the stability of C₂R₂ in high ionic strength buffers was obtained from exchange experiments. When C₂R₂ (0.1 μ M) was incubated with *C (0.36 μ M) in 50 mM Tris-HCl containing 2 mM 2-mercaptoethanol at pH 7.5, the amounts of radioactivity incorporated into C₂R₂ after 0, 5, 70, and 120 min were 4.0, 4.0, 5.0, and 5.0%, respectively. In similar experiments in

^f Evans *et al.* (26) reported that C₂R₂ disproportionates about 70% in 6 min in 50 mM Tris-HCl at pH 9.0 and about 80% in 6 min at pH 10.0–10.5. In comparable solutions containing 2 mM 2-mercaptoethanol we observed less than 10% disproportionation of C₂R₂ in 2 hr. When saturating amounts of PALA were present, 40% of the C₂R₂ disproportionated in the same time interval.

FIG. 2. Kinetics of disproportionation of C_2R_2 in the absence and presence of PALA, and the effect of temperature. Experiments were conducted as described in the legend to Fig. 1 except that reactions were stopped at designated times by adding concentrated buffer prior to loading the polyacrylamide gels. Results given as percent of C_2R_2 remaining at different times. (A) Experiments performed at 25°. Disproportionation in (Δ) absence and ($\bullet$, $\blacksquare$) presence of PALA (55 μ M). (B) Experiments at different temperatures with PALA present at 55 μ M.

which PALA was present (33 μ M) the incorporation was 2.7, 2.3, and 2.3% after 0, 30, and 60 min, respectively.

The marked stability of C_2R_2 in the high ionic strength buffer afforded a method for stopping the disproportionation process conducted with the solutions of lower ionic strength. When *C was present during the disproportionation process (at low ionic strength), large amounts of radioactivity disappeared from the *C band and were found in *C_2R_2 and *C_2R_3 (S. Subramani and H. K. Schachman, unpublished). Based on this observation, the effectiveness and the rapidity of stopping the kinetic process were tested by examining the extent of incorporation of *C into C_2R_2 within 5, 10, 15, and 20 sec after the addition of concentrated buffer to give a final concentration of 50 mM Tris-HCl containing 2 mM 2-mercaptoethanol at pH 7.5. Regardless of the extent of the disproportionation process, i.e., different periods of time at the low ionic strength, more than 80% of the radioactivity was always found as *C . Moreover, no decrease in radioactivity as *C was observed in the interval between 5 and 20 sec. Analysis of the gels for the total amount of C_2R_2 , C_2R_3 , and C along with those from a control experiment without *C showed that the distribution of products was not affected by the addition of *C after the ionic strength was increased. The lack of significant incorporation of *C into C_2R_2 or C_2R_3 showed that the disproportionation was effectively stopped within about 5 sec after the increase in ionic strength.

Effect of Active Site Ligand on Rate of Disproportionation of C_2R_2 . The marked effect of PALA on the rate of conversion of C_2R_2 into C_2R_3 and C is shown in Fig. 2A. In the absence of PALA less than 5% of the C_2R_2 had disproportionated in 2 hr. When PALA was present, however, about 80% of the C_2R_2 was converted into products in about 20 min. This rapid disproportionation was then followed by a very slow process over the next 100 min, leading to an additional conversion of about 5%.

Effect of Temperature on Ligand-Promoted Rate of Disproportionation of C_2R_2 . As seen in Fig. 2B the rate of disproportionation increased markedly when the temperature of the reaction mixture was raised from 0° to 35°. Moreover, the extent of the conversion also increased at the higher temperature.

DISCUSSION

As seen in Tables 1 and 2 there was virtually no exchange when free *C and *R subunits were incubated with native ATCase for 2 hr. Only *ATCase modified randomly by reacting some of its amino groups with an imidoester (labeled with ^{125}I)

showed any tendency to exchange in 2 hr with free native R subunits. This slight exchange was detected only in low ionic strength solutions containing the active site ligand, PALA. The back exchange with *ATCase might be favored over the forward exchange since the labeled enzyme molecules could be destabilized slightly as a result of the chemical modification of the C and R subunits. In contrast, the exchange with *R subunits and native ATCase would be expected to be less likely since the modified free subunits would have to compete with the native subunits within the intact enzyme. Although these exchange experiments with *ATCase are of potential use in efforts to determine the strength of the c - r bonding domains in the enzyme, we found it advantageous to focus on the less stable species, C_2R_2 , which disproportionates to form ATCase and free C subunits (Fig. 1).

The disproportionation process may be represented as $3 C_2R_2 \rightarrow 2 C_2R_3 + 2 C$. The proposed stoichiometry of this reaction has not been established quantitatively. The products are clearly ATCase (C_2R_3) and free C subunits; moreover, the amounts of C_2R_3 and C formed in the process are approximately equal (as determined from the gels) and the rates of disappearance of C_2R_2 (plotted in Fig. 2) correspond closely to the rates of formation of C_2R_3 and C . As seen in Fig. 2, the disproportionation of C_2R_2 does not proceed to completion even at times longer than 2 hr. A complex series of reactions is involved in the process (33); the mechanism of the process and a test of it will be presented in detail elsewhere (S. Subramani and H. K. Schachman, unpublished). Both the rate and the extent of the disproportionation are decreased by the addition of free C subunits, whereas the addition of C_2R_3 has no effect (33). These observations are readily interpreted in terms of a mechanism involving three different reactions to account for the observed rate of disappearance of C_2R_2 . First, some C_2R_2 molecules dissociate into free C and R subunits (a sequence of reactions is implicated in this dissociation). Second, some R subunits formed early in this process combine with remaining C_2R_2 molecules to form C_2R_3 (24). Finally, when the concentration of C_2R_2 becomes low, the free R subunits combine instead with free C subunits to form both C_2R_2 and C_2R_3 (24). This reformation of C_2R_2 and C_2R_3 from free subunits during the disproportionation was demonstrated readily by measurements of the incorporation of ^{125}I present initially in the solution as free *C (33).

According to this model, measurements of the kinetics of disappearance of C_2R_2 should provide quantitative values for the rate of rupture of the c - r bonding domains in C_2R_2 . The rates of all the reactions described above have not been deter-

mined and thus quantitative estimates of the rate of rupture of the c-r bonding domains must be considered tentative. A preliminary value is obtained by assuming that the disappearance of C_2R_2 due to its combining with free R is compensated by formation of C_2R_2 from free subunits. According to this assumption, the half-time for the disappearance of C_2R_2 would give a first-order rate constant for the rupture of two c-r bonding domains. The values per two c-r bonding domains calculated in this way from the data in Fig. 2 were $3.5 \times 10^{-6} \text{ sec}^{-1}$ for C_2R_2 in the absence of any ligand and $1.2 \times 10^{-3} \text{ sec}^{-1}$ for C_2R_2 in the presence of PALA. Although these values may be subject to revision when other rate constants are determined, it is clear that the rate of disproportionation of C_2R_2 , and hence the rate of rupture of a c-r bonding domain, is enhanced several hundredfold when PALA is added to C_2R_2 in solutions of low ionic strength.⁸

Many of the kinetic properties of ATCase and the ligand-promoted conformational changes in the enzyme (20) can be accounted for in terms of the two-state model of Monod *et al.* (1). According to this model, the enzyme exists in an equilibrium between a constrained conformation having a low affinity for substrates and a relaxed form of higher affinity. The addition of the bisubstrate analog, PALA, to ATCase causes a decrease in the sedimentation coefficient of the enzyme and a marked enhancement in the reactivity of the sulfhydryl groups on the R subunits (20). Both of these effects, which are attributed to a ligand-promoted conformational change of the enzyme to the relaxed conformation, are observed with C_2R_2 (S. Subramani and H. K. Schachman, unpublished). The data presented here show that the PALA-promoted allosteric transition can be correlated with the weakening of the c-r bonding domains. This weakening, based on the rate constants presented above and the rate of formation of the c-r bonding domain (24), which is assumed to be the same for both conformational states, corresponds to about 1.7 kcal/mole (7.1 kJ/mole) per c-r bonding domain.

In spite of the similarity between C_2R_2 and ATCase in terms of enzyme kinetics and ligand-promoted changes in physical properties, it is important to recognize that the c-r bonding domains in these two types of molecules may differ in strength. Under conditions where C_2R_2 disproportionates rapidly (low ionic strength and the presence of PALA), ATCase exhibits no dissociation and only slight exchange of subunits. Hence the c-r bonding domains in ATCase appear to be stronger than in C_2R_2 . Finally, our demonstration of the weakening of the c-r bonding domains in C_2R_2 upon the addition of the active-site ligand does not preclude either the weakening or strengthening of the r-r or c-c domains in these molecules. A complete description of the allosteric transition requires measurements of the effect of ligands on these bonding domains as well. In addition, an appropriate solvent must be found so that enzyme kinetics, conformational changes, and the strengths of the bonding domains can be measured under similar conditions. The techniques

presented here in preliminary form show promise for such investigations.

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⁸ Disproportionation of C_2R_2 was also enhanced in solutions containing both carbamoyl phosphate and succinate. Since these active-site ligands bind to the protein much less strongly than the bisubstrate analog, PALA, the saturation of the active sites by carbamoyl phosphate and succinate requires relatively high concentrations. Thus their effect on accelerating the disproportionation of C_2R_2 by weakening the c-r bonding domains is partially masked as a result of the increase in the ionic strength of the solution.