Allosteric Interactions in Aspartate Transcarbamylase. I. Binding of Specific Ligands to the Native Enzyme and Its Isolated Subunits*

J.-P. Changeux,† J. C. Gerhart, and H. K. Schachman

ABSTRACT: The regulatory enzyme, aspartate transcarbamylase (ATCase), which in earlier studies was shown to be composed of two catalytic and four regulatory subunits, was examined with regard to its stereospecific interactions with ligands. Equilibrium dialysis experiments reveal four specific binding sites for succinate (an analog of the substrate, aspartate) and four specific sites for the feedback inhibitor, cytidine triphosphate (CTP) or its analog, 5-bromocytidine triphosphate (BrCTP) per molecule of enzyme. On the basis of these results and evidence from dissociation studies, ATCase is viewed as an isologous tetramer, the protomers of which each contain one regulatory subunit (mol wt 2.7×10^4) and one-half of a catalytic subunit (mol wt 5.0×10^4). It was found that the isolated regulatory subunit possessed one binding site for CTP and the isolated catalytic subunit (mol wt 1×10^5) possessed two sites for succinate. Whereas the binding of ligands to the isolated subunits is normal, unusual effects are observed for the binding of these same ligands to the native enzyme. ATCase exhibits *cooperative* effects for succinate binding as revealed by a sigmoidal saturation curve (with a Hill coefficient of 1.6) and *antagonistic* effects as revealed by a partial reduction of CTP binding by succinate. It is concluded that the binding of succinate and CTP occurs at topographically distinct sites derived exclusively from the folded polypeptide chains of the different subunits, and that the *cooperative* and *antagonistic* effects are therefore indirect, *i.e.*, allosteric effects, which must be mediated by the protein itself. In contrast to these indirect effects, ATCase also exhibits a direct effect in which the inhibitor (CTP) and the activator (adenosine triphosphate, ATP) appear to compete for a single site on the regulatory subunit.

Among the many regulatory enzymes which have been examined thoroughly by kinetic analysis, aspartate transcarbamylase (ATCase)[1] from *Escherichia coli* is one of the few which also has been characterized extensively in terms of its physical and chemical properties. ATCase catalyzes the initial reaction unique to pyrimidine biosynthesis (the carbamylation of aspartate by carbamyl phosphate) and is inhibited by cytidine triphosphate (CTP), an end product of the pyrimidine pathway (Yates and Pardee, 1956; Gerhart and Pardee, 1962). This sensitivity of ATCase to CTP establishes in *E. coli* the familiar regulatory pattern of feedback inhibition. From the kinetic studies, it has been concluded that the native enzyme exhibits two classes of indirect, *i.e.*, allosteric, interactions (Monod *et al.*, 1963) among the specific ligands involved in the regulation of its activity. On the one hand, ATCase exhibits *heterotropic*[2] effects between the substrate (aspartate) and either the feedback inhibitor (CTP) or the activator (ATP). On the other hand, ATCase (in the presence of the other substrate, carbamyl phosphate) exhibits *homotropic*[2] effects as evidenced by the sigmoidal dependence of reaction velocity on the concentration of aspartate (Gerhart and Pardee, 1962–1964; Gerhart, 1964). Since ATCase maintains these interactions even as a highly purified protein (Gerhart and Holoubek, 1967), which is stable and available in gram quantities

* From the Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720. *Received August 28, 1967.* These studies were supported in part by Public Health Service Research Grant CA 07410 from the National Cancer Institute and Research Grant GM 12159 from the National Institute of General Medical Sciences, U. S. Public Health Service; by National Science Foundation Research Grant GB-4810X; and by a contract between the Office of Naval Research and the Regents of the University of California.

† On leave from the Pasteur Institute, Paris, France. Dernham Fellow of the American Cancer Society, California Division, and Eleanor Roosevelt Fellow of the International Union Against Cancer.

[1] Carbamyl phosphate:L-aspartate carbamyl transferase (EC 2.1.3.2). Abbreviations used: ATCase, aspartate transcarbamylase; CTP, cytidine triphosphate; ATP, adenosine triphosphate; BrCTP, 5-bromocytidine triphosphate; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene.

[2] The nomenclature used in this paper was proposed by Monod *et al.* (1965). (1) *Allosteric effects* are defined as *indirect* interactions between topographically *distinct* binding sites mediated by the protein molecule through a conformational transition. Heterotropic interactions occur between dissimilar ligand molecules and are typified by the effect of activators and inhibitors on the turnover number or apparent affinity (or both) of the enzyme for its substrate. Homotropic interactions occur between identical ligand molecules and are ex-

and can be dissociated into separable catalytic and regulatory subunits (Gerhart, 1964; Gerhart and Schachman, 1965), it constitutes a choice material for an analysis of the elements and dynamics of structure responsible for the allosteric interactions characteristic of regulatory enzymes.

This paper presents equilibrium dialysis measurements on the binding of specific ligands to the native enzyme and to the isolated catalytic and regulatory subunits. In agreement with the inference from the kinetic studies, the binding data demonstrate directly that ATCase mediates homotropic interactions revealed by the cooperative binding of the substrate analog, succinate, and heterotropic interactions revealed by the antagonistic effect of CTP on succinate binding. Moreover, these interactions are lost when the ATCase molecule is dissociated into subunits even though the catalytic subunit still binds the substrate analog and the regulatory subunit still binds the feedback inhibitor. Data on the identity and the number of stereospecific sites are consistent with a tetrameric structure of the native enzyme.

Experimental Procedures

Materials

Enzyme. Native ATCase and the isolated catalytic and regulatory subunits were prepared as described by Gerhart and Holoubek (1967).

Chemicals. [^{14}C]Succinate and cytidine [2-^{14}C]-5′-triphosphate (specific activity 9.1 and 20.5 c/mole, respectively) were purchased from Schwarz Bio-Research, Inc. CTP and ATP were purchased from Sigma Chemical Co. Using the method of Bessman *et al.* (1958), 5-bromocytidine triphosphate (BrCTP) and [^{14}C]BrCTP were synthesized from unlabeled and labeled CTP, respectively. BrCTP had an absorption maximum of 299 mμ at pH 2 with an extinction coefficient taken as 9.2×10^3 M^{-1} (Bessman *et al.*, 1958) and an absorption ratio at 280:260 mμ of 3.5. Dilithium carbamyl phosphate was obtained from Sigma Chemical Co. and purified further by precipitation from a 25-mg/ml solution in cold water by the addition of an equal volume of cold ethanol (Gerhart and Pardee, 1962). Aqueous stock solutions (8 mg/ml) of dilithium carbamyl phosphate were stored at −15°.

Scintillation fluid contained 4.61 g of PPO and 0.115 g of dimethyl-POPOP/kg (1.15 l.) of toluene. Scintillators were obtained from Packard Co., as was Hyamine hydroxide (1 M in methanol).

Buffers. Equilibrium dialysis experiments were performed with buffered solutions containing 4×10^{-2} M potassium phosphate (pH 7.0), 2×10^{-3} M 2-mercaptoethanol, and 2×10^{-4} M Versene, supplemented when indicated with 2×10^{-2} M Tris (pH 7.0) and 8×10^{-3} M carbamyl phosphate.

Measurement of Ligand Binding

Preliminary ligand binding studies were performed using a sedimentation velocity technique in which unbound ligand was measured spectrophotometrically (Schachman *et al.*, 1962; Lamers *et al.*, 1963). Most of the experiments were performed by equilibrium dialysis with radioactive ligands (Myer and Schellman, 1962). The use of radioactive ligands in the latter technique obviated the need for spectral information about the ligands and allowed the detection of much lower ligand concentrations than was possible spectrophotometrically.

Dialysis was performed in cylindrical lucite cells constructed according to the design of Myer and Schellman (1962) and having a total capacity of 0.8 ml which was divided into two equal compartments by a semipermeable membrane of 0.5-cm radius. The membranes were cut from cellulose dialysis tubing (Visking C°) which had been washed for 6 hr in distilled water, dehydrated in increasing concentrations of ethanol, and stored under 95% ethanol. Prior to use, the membranes were rehydrated in distilled water and equilibrated overnight against buffer. With these membranes, the half-time for transport (at 21°) was approximately 3 hr for succinate and 8 hr for CTP or BrCTP. In the presence of protein at high concentrations, these times were slightly longer. Dialysis rates were increased by treatment of the membranes with $ZnCl_2$ (Craig and King, 1962). Strips of hydrated membranes (10 × 2 cm) were incubated at room temperature in petri dishes containing an aqueous solution of $ZnCl_2$ which had a density of 1.8 g/ml. The dishes were agitated slightly to prevent extended contact of the membranes with the glass. Under these conditions a 10-min treatment was found to reduce the equilibration time for ligand dialysis without rendering the membrane permeable to protein.

In a typical experiment 0.3 ml of enzyme solution (5–20 mg of protein/ml) which had been dialyzed against buffer was placed in one compartment of each dialysis cell and 0.3 ml of radioactive ligand in the same buffer was placed in the other compartment. The total ligand concentration was varied from one cell to another, but the amount of radioactivity was maintained at 10^4 cpm/cell. After equilibration, 0.05-ml aliquots of solution were removed from both compartments and

pressed by the sigmoidal saturation curve of the enzyme by the considered ligand. (2) *Quaternary structure.* A polymeric protein containing a finite, relatively small number of identical subunits is said to be an *oligomer*, if the number of identical subunits is unspecified, or a dimer, trimer, etc., if the number is specified. The identical subunits (*i.e.*, the repeated units) associated within an oligomer are designated as *protomers.* The term "subunit" is purposely undefined and may be used to refer to any chemically or physically indentifiable submolecular entity within a protein. (3) *Association of protomers.* The spatially arranged collection of all groups (*i.e.*, amino acid side chains) on one protomer which are involved in its binding to another protomer is termed a *binding set.* Considered together, the two binding sets through which two protomers associate are called the *domain of bonding* of the pair. Two modes of association between protomers have been distinguished. (a) When the domain of bonding is composed of two different binding sets, the protomers are said to engage in *heterologous association.* (b) When the domain of bonding is composed of two identical binding sets, the protomers are said to engage in *isologous association.* Association between different (nonprotomeric) subunits is always heterologous.

each was mixed with 1 ml of a commercial preparation of 1 M Hyamine hydroxide in methanol and then with 10 ml of scintillation fluid. Samples were counted with a Nuclear-Chicago Mark I liquid scintillation counter. Concentrations of free ligand (C_F) and of bound ligand (C_B) were determined from the formulas

$$C_F = \frac{C_I D_F}{D_P + D_F}; \quad C_B = \frac{C_I(D_P - D_F)}{D_P + D_F}$$

where C_I is the initial molar concentration of ligand in one compartment of the cell and D_P and D_F are the counts per minute of the aliquots containing and lacking protein, respectively. In all experiments, the ligand introduced initially was accounted for within 5% by measurements of D_P and D_F, and the initial and final protein concentrations in the one compartment were the same.

Protein concentrations were estimated spectrophotometrically. The absorbances at 280 mμ (path length, 1 cm) of 0.1% solutions of native enzyme and catalytic subunit are 0.59 and 0.72, respectively, at pH 7 (Gerhart and Schachman, 1965). Concentrations of regulatory subunit were routinely measured by the method of Folin and Ciocalteau as described by Lowry *et al.* (1951). Values taken for the molecular weight were 3.0×10^5 for native enzyme and 1.0×10^5 for catalytic subunit (Gerhart and Schachman, 1965). A definitive molecular weight has not yet been determined for the regulatory subunit (estimates presently range from 2.5 to 3.5×10^4) because of its tendency for aggregation. A minimum estimate corresponding to the weight of regulatory subunit per mole of bound feedback inhibitor was therefore determined from the equilibrium dialysis data.

Results

Effects of Carbamyl Phosphate on Succinate Binding. In the present binding studies, succinate is used as a specific ligand for the active site since it is an unreactive analog which has been shown to be a simple competitive inhibitor of aspartate (Gerhart and Pardee, 1964). Results shown in Table I demonstrate that the binding of succinate to both the native enzyme and the isolated catalytic subunit is dependent upon the presence of carbamyl phosphate.[3] In light of this finding, all the experiments on the binding of succinate were performed in the presence of saturating concentrations of carbamyl phosphate (more than 2×10^{-3} M).

Succinate Binding to the Native Enzyme. Figure 1a illustrates that the binding of succinate to native ATCase, in the presence of carbamyl phosphate, does not follow the simple Langmuir isotherm. Instead, when the amount of succinate bound to the enzyme is plotted as a function of free succinate concentration, a sigmoidal curve is obtained. This unusual binding behavior is illustrated further when the same data are plotted according to Scatchard (1949). As seen in Figure 1b, the data yield a complex curve with a maximum, and not the straight line characteristic of ligand binding to a multivalent protein with identical and independent sites (Klotz, 1953). In addition, a plot of the data according to Hill (Brown and Hill, 1922) yields a curve with a maximum slope of 1.6 (Changeux and Rubin, 1968). This slope differs significantly from the value of 1.0 expected for noncooperative binding. These observations show directly that cooperative homotropic interactions in ligand binding are mediated by the native enzyme, a conclusion inferred earlier from kinetic studies (Gerhart and Pardee, 1962).[4]

TABLE I: Requirement for Carbamyl Phosphate in the Binding of Succinate to Native ATCase and the Catalytic Subunit.[a]

Protein	Carbamyl Phosphate (M × 10^3)	r[b]
Native ATCase	0	≤0.09
	4	0.87
Catalytic Subunit	0	≤0.05
	4	0.51

[a] Equilibrium dialysis was conducted by the technique of Myer and Schellman (1962), using [^{14}C]succinate as described in the Experimental Procedures. Dialysis time at 21° was 12 hr. [b] Values for the amount of succinate bound are expressed as r, the average number of succinate molecules bound *per molecule* of native enzyme (mol wt 3.1×10^5) or catalytic subunit (mol wt 1.0×10^5).

The number of succinate binding sites is given by the intercept with abscissa of the extrapolated linear part of the Scatchard plot (corresponding to infinite succinate concentration). The value obtained is 3.8 ± 0.2 sites per mol wt 3.0×10^5. Under these conditions half-saturation of the substrate binding sites is obtained at a concentration of free succinate of $6.3 \pm 0.2 \times 10^{-4}$ M.

Succinate Binding to the Catalytic Subunit. In contrast to results with the native enzyme, the Scatchard plot for succinate binding to the catalytic subunit yields a straight line (Figure 1c). Even though the number of sites per catalytic subunit is 1.8 ± 0.2 per 1.0×10^5 mol wt, it appears that no homotropic interactions occur for succinate binding to the catalytic subunit. The

[3] This observation, which is presumably related to the ordered addition of substrates required for the carbamylation of aspartate, will be treated elsewhere.

[4] The qualitative rather than quantitative agreement between the binding results and the kinetic data of Gerhart and Pardee (1963) may be due to unavoidable differences in experimental conditions (particularly the protein concentration) or to velocity effects inherent in kinetic studies.

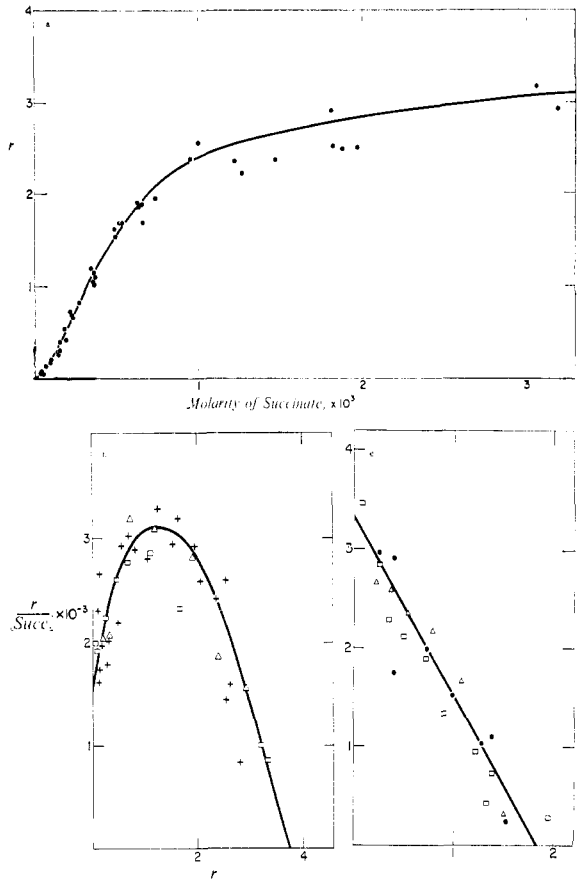

FIGURE 1: Binding of succinate to ATCase and to the catalytic subunit. Data are expressed as r, the moles of succinate bound per mole of ATCase or catalytic subunit. Part a shows the binding to ATCase plotted as r *vs.* the concentration of free succinate. The same data appear in b as a Scatchard plot, r/[free succinate] *vs.* r. Part c is a Scatchard plot for the binding of succinate to the catalytic subunit. Equilibrium dialysis was conducted by the technique of Myer and Schellman (1962) using [^{14}C]succinate as described in Experimental Procedures. Carbamyl phosphate was present at 4×10^{-3} M. ATCase in a and b was present in the protein compartment of the dialysis cell at a concentration of 7.5×10^{-5} M (22.5 mg/ml); catalytic subunit in c was present at 1.0×10^{-4} M (10.0 mg/ml). Dialysis time at 21° is indicated by the symbols + and ● for 12 hr, Δ for 16 hr, and □ for 20 hr.

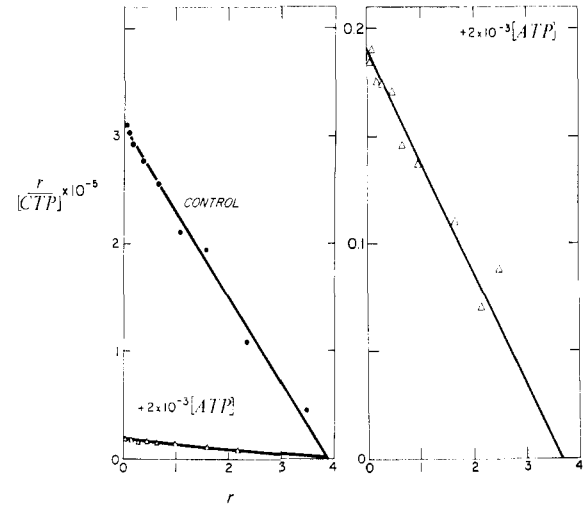

FIGURE 2: Binding of CTP to ATCase and the effect of ATP on CTP binding. Data are given as a Scatchard plot with r representing the moles of CTP bound per mole of ATCase. In a data are shown for the binding of CTP in the absence (●) and in the presence of ATP (Δ) at a concentration of 2×10^{-3} M. Part b gives the same data for the binding of CTP in the presence of ATP with the ordinate magnified 20-fold. Equilibrium dialysis was conducted by the technique of Myer and Schellman (1962) using [^{14}C]CTP. No carbamyl phosphate was present. ATCase concentration in the protein compartment of the dialysis cell was 6.1×10^{-5} M (18.3 mg/ml). Dialysis time at 21° was 48 hr.

intrinsic dissociation constant determined from the Scatchard plot is $5.5 \pm 0.2 \times 10^{-4}$ M. The finding of approximately two sites per catalytic subunit is consistent with the value (Figure 1b) of about four sites per native ATCase molecule, and the presence of two catalytic subunits per native enzyme molecule (Gerhart and Schachman, 1965).

CTP and BrCTP Binding to the Native Enzyme. With both the ultracentrifugal technique of Schachman *et al.* (1962) and the equilibrium dialysis method of Myer and Schellman (1962), it was found (Figure 2) that there are 3.8 ± 0.1 binding sites for the feedback inhibitor CTP per native enzyme molecule of 3.0×10^5 mol wt. Similar results are obtained for BrCTP, a structural analog of CTP. As shown in Figure 2, the Scatchard plot for the binding of CTP is linear over a wide range of concentrations; however, deviations from linearity were observed in several experiments at high fractional saturations ($r = >3.5$). It is not yet clear whether the observed binding at high ligand concentrations is in error due to an artifact of the binding technique or whether it includes binding to secondary sites with lower affinity.[5] In the present investigation, only sites exhibiting high affinity are considered. It is noteworthy that cooperativity in the binding of CTP is not apparent, as evidenced by the linear Scatchard plots. The intrinsic dissociation constants for CTP and BrCTP are 1.3 and $1.1 \pm 0.1 \times 10^{-5}$ M, respectively.

Inhibitor Binding to the Regulatory Subunit. Studies by equilibrium dialysis confirmed the earlier sedimen-

[5] In earlier studies (Gerhart and Schachman, 1965) eight combining sites were found for BrCTP at a different pH and ionic strength. These experiments were conducted at higher degrees of saturation of the enzyme. The present findings indicate four principal binding sites and further work is in progress to clarify these findings.

TABLE II: Reversal of BrCTP Binding to Native ATCase by Succinate.[a]

Total[b] M of Succinate ($\times 10^5$)	Total[b] M of BrCTP ($\times 10^5$)	BrCTP Bound (r)[c]	(r_s/r_0)[d]
0	0.10	0.033	
	0.72	0.24	
	11	2.82	
250	0.10	0.029	0.88
	0.72	0.21	0.88
	11	2.31	0.82
500	0.10	0.027	0.82
	0.72	0.21	0.79
	11	2.13	0.75

[a] Equilibrium dialysis was conducted by the technique of Myer and Schellman (1962) with [^{14}C]BrCTP as described in the Experimental Procedures. Dialysis time at 21° was 21 hr. Carbamyl phosphate was present at 4×10^{-3} M. [b] Total molarities of succinate and BrCTP were calculated from the millimoles of each added initially to the dialysis cell divided by the 0.6 ml, the total liquid volume in the cell. For succinate, the total molarity is approximately 20-fold (or greater) in excess of the succinate binding sites and is therefore approximately equal to the free concentration of succinate. [c] Values for the amount of BrCTP bound are given as r, the number of BrCTP molecules bound per molecule of ATCase. [d] This ratio represents the binding of BrCTP in the presence of succinate (r_s) relative to the binding of BrCTP in the absence of succinate (r_0).

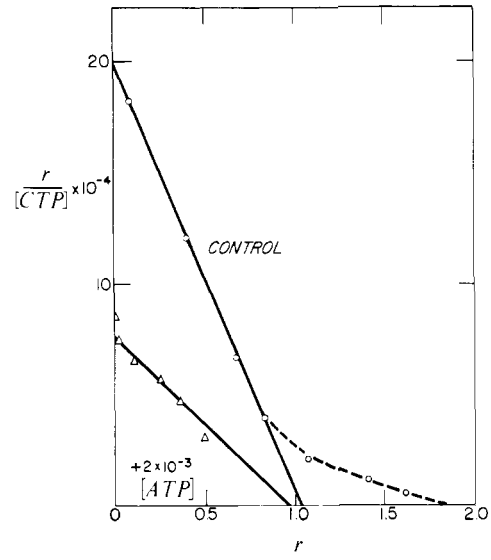

FIGURE 3: Binding of CTP to the regulatory subunit and the effect of ATP on CTP binding. Data are given as a Scatchard plot with the symbol r indicating the moles of CTP bound per mole of regulatory subunit with an assumed molecular weight of 2.7×10^4. Binding of CTP in the absence of ATP is symbolized by (○) and in the presence of 2×10^{-3} M ATP is symbolized by (△). The same conditions were used as described in Figure 2 except that the concentration of regulatory subunit was 3.2 mg/ml and the temperature was 4° (to retard the denaturation of the subunit).

tation velocity experiments showing that CTP (or BrCTP) is bound firmly to the isolated regulatory subunit. The data were analyzed in the form of a Scatchard plot (Figure 3) in order to obtain a combining weight in molecular weight units of protein corresponding to one CTP binding site. This combining weight was calculated to be 2.7×10^4, a value consistent with the molecular weight of 3×10^4 determined from sedimentation equilibrium experiments (Gerhart and Schachman, 1965). Moreover, this result is consistent with the finding of four CTP binding sites per ATCase molecule (Figure 2) since it was shown previously (Gerhart and Schachman, 1965) that there are four regulatory subunits (each of mol wt 3×10^4) per ATCase molecule. The dissociation constant for CTP bound to the regulatory subunit is $5.1 \pm 0.1 \times 10^{-5}$ M (at 4°). As seen in Figure 3 there appears to be additional binding at high CTP concentrations. If this apparent binding is attributed to sites of lower affinity, the dissociation constant for them would be about tenfold greater than for the high affinity sites mentioned above.

Antagonism of Inhibitor Binding by Succinate. In the presence of succinate the amount of BrCTP bound to native ATCase is reduced. The antagonism, as seen in Table II, is only partial and cannot result from simple competition between succinate and BrCTP for the same sites on the enzyme. This conclusion is based on a comparison of the binding results for different ratios of succinate to BrCTP. For example, when the succinate concentration (2.5×10^{-3} M) is 23 times that of the BrCTP (1.1×10^{-4} M), the relative binding of the latter is 82% that of the control (with no succinate); similarly when the succinate concentration (5×10^{-3} M) is 5000-fold greater than that of BrCTP (1×10^{-6} M), the binding of BrCTP is still 82% that of the control (with no succinate). Although in these experiments it was not possible to measure directly the fractional saturation of the enzyme by succinate (in the presence of BrCTP), it can be inferred from the kinetic studies of Gerhart and Pardee (1964) that at the high levels of succinate used herein (ten times that required for half-saturation) the BrCTP-inhibited enzyme was nearly saturated by succinate. It would appear, therefore, that BrCTP and succinate can bind simultaneously to the enzyme and that the binding sites for the substrate analog and the inhibitor, which are contained on different subunits, do not overlap in the native enzyme.

Antagonism of Inhibitor (CTP) Binding by the Activa-

tor (ATP). In addition to the substrates and inhibitors of ATCase, another class of ligands (activators) has been identified by kinetic methods (Gerhart and Pardee, 1962). These ligands, such at ATP, reverse the inhibition of the enzyme by CTP and even in the absence of CTP increase the apparent affinity of the enzyme for the substrate, aspartate.

As seen in the equilibrium dialysis experiments with the native enzyme (Figure 2), the binding of CTP is greatly reduced when ATP is added. Comparable experiments with the regulatory subunits show that the binding of CTP is also markedly decreased by the addition of ATP (Figure 3). The antagonism between ATP and CTP is thus preserved when the regulatory subunit is isolated from the native enzyme molecule.

Furthermore, Figure 4 shows that with the native enzyme the antagonism between ATP and CTP is competitive. The values of $(CTP)[(4 - r_{CTP})/r_{CTP}]$ vary linearly with ATP concentration and thus at infinite ATP concentration CTP can be completely displaced by ATP.[6] These observations strongly suggest a mutual exclusion between ATP and CTP at the same site on the regulatory subunit.

On the assumption of direct competition the dissociation constant for ATP bound to the native enzyme is calculated to be 1.2×10^{-4} M (at 21°) (Figure 4). A corresponding value of 1.1×10^{-4} M is obtained from experiments in which the ATP concentration was fixed and the CTP varied (Figure 2). With the regulatory subunit, the dissociation constant for ATP is calculated to be 1.5×10^{-3} M (at 4°).

Discussion

The Interactions of Ligands Binding to ATCase and Its Isolated Subunits. As the equilibrium dialysis experiments show, ATCase binds specifically various small molecules including the substrate (carbamyl phosphate), a nonreacting substrate analog (succinate), and two effectors (CTP and ATP), which regulate the catalytic activity of the enzyme as an inhibitor and an activator, respectively. The binding properties of ATCase are unusual in that the binding of any specific ligand is accompanied by marked effects on the subsequent binding of additional ligands. These effects comprise enhancement or antagonism of ligand binding and they may occur in multiple binding of the same ligand (*i.e.*, homotropic effects) or in the binding of dissimilar ligands (*i.e.*, heterotropic effects). ATCase affords an unusual opportunity to clarify the nature of these effects since some are dependent on the integrity of the native structure of the enzyme whereas others are manifested as well by the separated catalytic and regulatory subunits. For the following discussion the different effects are divided into two categories: the first comprising those effects which require the participation of only a single subunit and the second, those effects requiring the participation of multiple subunits integrated in the native enzyme molecule.

Effects included in the first category are: (1) the marked enhancement of succinate binding by the presence of carbamyl phosphate, observed both for the catalytic subunit and for the intact ATCase molecule. Probably the interaction between these two compounds occurs at a topographically close region within a single active site and is important for the carbamylation reaction (when the substrate, aspartate, is used). The observed enhancement may involve a preferred order of binding of carbamyl phosphate and succinate to the enzyme, in which case ATCase would resemble other enzymes which exhibit ordered addition of substrates.[7] (2) The *antagonism* between the *activator* (ATP) and the *inhibitor* (CTP) is found both for ATCase and for the regulatory subunit. This antagonism follows the simple relationship for competition at a single binding site. Thus we can conclude that the binding sites for CTP and ATP on the regulatory subunits of the enzyme are either identical or sufficiently close to permit mutual ligand exclusion simply by a direct effect such as *steric hindrance*.

The second category comprises effects which require the intact structure of the enzyme and are lost completely when the enzyme is dissociated into subunits. These effects include the antagonism of CTP binding by succinate,[8] a heterotropic effect, and the cooperative binding of succinate, a homotropic effect. The results from the equilibrium dialysis experiments provide clarification of the nature of these effects. (1) With regard to the heterotropic effect two findings are relevant.

[6] If one assumes simple competition between ATP and CTP for binding sites on ATCase, then one obtains the following relationship between r_{CTP} and ATP

$$(CTP)\frac{4 - r_{CTP}}{r_{CTP}} = \frac{K_C}{K_A}(ATP) + K_C$$

where K_C and K_A are the dissociation constants of the protein complexes containing CTP or ATP, respectively. This relationship takes into account the changes of concentration of unbound CTP which occur (a sixfold change) due to the displacement of CTP from ATCase by ATP.

[7] Reciprocal equilibrium dialysis experiments would be expected to reveal enhanced binding of carbamyl phosphate in the presence of succinate. The preferred order of binding, as well as the mechanism for ordered binding, requires kinetic measurements for substantiation. Various schemes (not necessarily involving sequential binding) may be visualized for the enhancement by carbamyl phosphate of succinate binding to ATCase. For example, one substrate might bind and contribute to the local environment (*e.g.*, charged groups) for the binding of the second substrate, without any concomitant alterations in the protein conformation. This direct effect between substrates is to be contrasted with a second possible scheme in which the protein preexists in an equilibrium mixture of various conformations, one of which binds both substrates strongly (an application of the model of Monod *et al.*, 1965). Addition of one of the substrates would shift the equilibrium distribution among the protein molecules and thereby lead to enhanced binding of the second substrate. A variation of this scheme has been proposed by Koshland (1964) in which the first substrate induces a change in conformation of the protein, leading to the unfolding of a binding site for the second substrate.

[8] Succinate binding was markedly reduced when CTP was present, but the data are not given here because it was not possible to obtain a reliable saturation curve over a wide range of succinate concentration.

The antagonism of CTP binding by succinate (Table II) is only partial thereby indicating that both CTP and succinate can bind simultaneously to ATCase at nonoverlapping sites. In addition the separated regulatory and catalytic subunits have high affinities for CTP and succinate, respectively. Since these affinities are not dramatically lower than those exhibited by the intact enzyme, it can be concluded that the critical tertiary structures required for binding are already attained by the individual subunits. (2) In the homotropic effect it is important to note that the two sites on each isolated catalytic subunit act independently in the binding of succinate (Figure 1c). Cooperative effects are exhibited only when two catalytic subunits are integrated into the native enzyme molecule.

These results indicate that the various specific binding sites on ATCase are topographically distinct and derived exclusively from the folded polypeptide chains of the separate subunits. Hence, the homotropic and heterotropic effects of the binding of one ligand on the subsequent binding of another must be indirect, *i.e.*, allosteric, effects and thus are mediated by the protein molecule (Monod *et al.*, 1963; Koshland, 1964). It is clear that the mediation of allosteric effects by ATCase definitely requires the native quaternary structure of the protein molecule.

The Oligomeric Structure of ATCase. The quantitative results of the binding experiments provide further information on the structure of the native enzyme. Since four binding sites for the substrate analog (succinate) and four sites for the feedback inhibitor (CTP (or BrCTP)) are found per molecule of native enzyme (mol wt 3.1×10^5), ATCase can therefore be visualized as a tetramer of *identical* units (protomers) which contain one site of each category.

Such a proposal of a tetrameric structure is not in contradiction with the earlier observation of Gerhart and Schachman (1965) that ATCase is composed of *unlike* subunits into which the enzyme dissociates in the presence of the mercurial, *p*-mercuribenzoate. Indeed, the various structural and binding data for ATCase can be incorporated into a topological model for the bonding between subunits, as recently proposed by Changeux *et al.* (1967). According to this model, ATCase is an isologous tetramer (defined by Monod *et al.*, 1965) of protomers, each of mol wt 7.7×10^4. One protomer would contain one regulatory subunit of mol wt 2.7×10^4 associated with one-half of a catalytic subunit of mol wt 5.0×10^5. This association within the protomer would occur through an area of bonding sensitive (directly or indirectly) to mercurial attack. Two protomers are associated through either their catalytic or regulatory moieties to form dimers, and identical dimers are associated to form the native isologous tetramer. The assignment of the mercurial sensitive bonding surface to a position *within* a protomer leads to the prediction that PMB causes an *asymmetric* split of ATCase into catalytic and regulatory subunits, as is found. The fact that the catalytic subunits are found to exist as dimers (each having two substrate binding sites) whereas the regulatory subunits exist largely as monomers (each possessing a single regulatory site), may merely reflect the difference in the strength of self-association of the isolated subunits. The existing data are thus consistent with an oligomeric structure for ATCase.

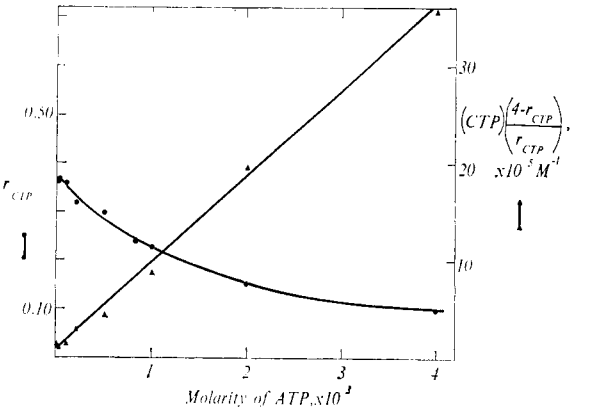

FIGURE 4: Antagonism of the binding of CTP to ATCase as a function of ATP concentration. The symbol (●) indicates the values of r (moles of CTP bound per mole of ATCase) as a function of free ATP concentration; the symbol (▲) indicates the values of $(CTP)[(4 - r_{CTP})/r_{CTP}]$ as a function of free ATP concentration. The experimental conditions were the same as described for Figure 2 except that the concentration of CTP was fixed (2.42×10^{-5} M CTP was added in the protein-free compartment of the dialysis cell at the beginning of the experiment) and the concentration of ATP was varied as indicated. ATCase concentration in the protein compartment of the dialysis cell was 6.0×10^{-5} M (10.0 mg/ml).

References

Bessman, M. J., Lehman, I. R., Adler, J., Zimmerman, S. B., Simms, E. S., and Kornberg, A. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 633.

Brown, W. E. L., and Hill, A. V. (1922), *Proc. Roy. Soc. (London) B94*, 297.

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1967), *in* Regulatory Mechanisms in Nucleic Acid and Protein Biosynthesis, Koningsberger, V. V., and Bosch, L., Ed., Biochimica Biophysica Acta Library, Vol. 10, Amsterdam, Elsevier, p 344.

Changeux, J.-P., and Rubin, M. (1968), *Biochemistry 7*, 553 (this issue; paper after following paper).

Craig, L. C., and King, T. P. (1962), *Methods Biochem. Anal. 10*, 175.

Gerhart, J. C. (1964), *Brookhaven Symp. Biol. 17*, 232.

Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242*, 2886.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

Gerhart, J. C., and Pardee, A. B. (1963), *Cold Spring*

Harbor Symp. Quant. Biol. 28, 491.
Gerhart, J. C., and Pardee, A. B. (1964), *Fed. Proc. 23*, 727.
Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.
Klotz, I. M. (1953), *Proteins 1*, 727.
Koshland, D. E. (1964), *Fed. Proc. 23*, 719.
Lamers, K., Putney, F., Steinberg, I. Z., and Schachman, H. K. (1963), *Arch. Biochem. Biophys. 103*, 379.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Monod, J., Changeux, J.-P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Myer, Y. P., and Schellman, J. A. (1962), *Biochim. Biophys. Acta 55*, 361.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.
Schachman, H. K., Gropper, L., Hanlon, S., and Putney, F. (1962), *Arch. Biochem. Biophys. 99*, 175.
Yates, R. A., and Pardee, A. B. (1956), *J. Biol. Chem. 221*, 757.

Allosteric Interactions in Aspartate Transcarbamylase. II. Evidence for Different Conformational States of the Protein in the Presence and Absence of Specific Ligands*

John C. Gerhart and Howard K. Schachman

ABSTRACT: In the previous paper it was shown that the antagonistic and cooperative effects observed in the multiple binding of ligands by aspartate transcarbamylase (ATCase) are indirect. In order to determine whether these allosteric effects are mediated by the protein itself through changes in its tertiary or quaternary structure, we conducted physical–chemical studies of the conformational state of the enzyme both in the presence and absence of specific ligands such as substrates or feedback inhibitors. The effect of ligands on the reactivity of the sulfhydryl groups of the enzyme was examined as a probe for possible changes in the conformation of the enzyme. Spectrophotometric titration of ATCase with *p*-mercuribenzoate (PMB) yielded a reaction end point of 27 ± 1 PMB per ATCase molecule.

Upon the addition of PMB to form mercaptide complexes the enzyme dissociated into two catalytic and four regulatory subunits. In partially reacted mixtures the undissociated enzyme bound no PMB whereas the dissociated products were fully reacted. Moreover, in this *all-or-none* reaction of the sulfhydryl groups of the enzyme, virtually all of the PMB was bound to the regulatory subunits and practically none (5% or less) of the PMB was complexed to the catalytic subunits. Of the 32 half-cystines (found by cysteic acid analysis) in the intact ATCase molecules, 24–28 were in the four regulatory subunits and 8 in the two catalytic subunits. Although the rate of reaction of the isolated regulatory subunits with PMB was too rapid for measurement by conventional spectrophotometry, the rate for the intact enzyme was easily determined. Pseudo-first-order reaction velocity constants were determined for solutions containing the ligands at differing concentrations. The addition of both carbamyl phosphate and the substrate analog, succinate (which bind to the catalytic subunits), led to a sixfold increase in the rate of reaction of the sulfhydryl groups in the regulatory subunits of ATCase. Succinate alone had no effect, and the effect of carbamyl phosphate alone was much less than that resulting from the addition of both ligands. This enhancement of the reactivity of these sulfhydryl groups of ATCase was opposed by the addition of the regulatory metabolite, cytidine triphosphate (CTP), or its analog, 5-bromocytidine triphosphate (BrCTP). This antagonism between substrates and feedback inhibitor was highly specific and only partial in character. In the absence of carbamyl phosphate and succinate, CTP (and BrCTP) had almost no effect on the reactivity of ATCase toward PMB. Companion studies of the gross morphology of the enzyme were made by sedimentation velocity measurements. In the presence of both carbamyl phosphate and succinate there was a 3.6% reduction in the sedimentation coefficient of ATCase. Both ligands were required and the magnitude of the reduction was a function of the concentration of succinate (at a fixed concentration of carbamyl phosphate). As in the experiments on the reactivity of the sulfhydryl groups, CTP opposed the effect of carbamyl phosphate and succinate in reducing the sedimentation coefficient of ATCase; again the antagonism was only partial In the absence of the other ligands CTP had virtually no effect. The observed changes in the reactivity of sulfhydryl groups and in the hydrodynamic behavior appear to result from modification in the conformation of the undissociated protein and to be the indirect effect of ligands bound to the enzyme. It appears that in the presence of carbamyl phosphate and succinate the intact enzyme exists in a swollen (or anisometric) conformation which exhibits an enhanced reactivity toward PMB, perhaps because of its greater rate of dissociation into catalytic and regulatory subunits. When these ligands are absent, the intact enzyme appears to exist in a more compact conformation; the presence of CTP favors this compact, slowly reacting conformational state of ATCase.