Cooperative Binding of the Bisubstrate Analog N-(Phosphonacetyl)-L-aspartate to Aspartate Transcarbamoylase and the Heterotropic Effects of ATP and CTP

(Received for publication, August 8, 1988)

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Most investigations of the allosteric properties of the regulatory enzyme aspartate transcarbamoylase (ATCase) from Escherichia coli are based on the sigmoidal dependence of enzyme activity on substrate concentration and the effects of the inhibitor, CTP, and the activator, ATP, on the saturation curves. Interpretations of these effects in terms of molecular models are complicated by the inability to distinguish between changes in substrate binding and catalytic turnover accompanying the cooperative binding process. In an effort to eliminate this ambiguity, the binding of the 3H-labeled bisubstrate analog N-(phosphonacetyl)-L-aspartate (PALA) to aspartate transcarbamoylase in the absence and presence of the allosteric effectors ATP and CTP has been measured directly by equilibrium dialysis at pH 7 in phosphate buffer. PALA binds with marked cooperativity to the holoenzyme with an average dissociation constant of 110 nM. ATP and CTP alter both the average affinity of ATCase for PALA and the degree of cooperativity in the binding process in a manner analogous to their effects on the kinetic properties of the enzyme; the average dissociation constant of PALA decreases to 65 nM in the presence of ATP and increases to 266 nM in the presence of CTP while the Hill coefficient, which is 1.95 in the absence of effectors, becomes 1.35 and 2.27 in the presence of ATP and CTP, respectively. The isolated catalytic subunit of ATCase, which lacks the cooperative kinetic properties of the holoenzyme, exhibits only a very slight degree of cooperativity in binding PALA. The dissociation constant of PALA from the catalytic subunit is 95 nM. Interpretation of these results in terms of a thermodynamic scheme linking PALA binding to the assembly of ATCase from catalytic and regulatory subunits demonstrates that saturation of the enzyme with PALA shifts the equilibrium between holoenzyme and subunits slightly toward dissociation. Ligation of the regulatory subunits by either of the allosteric effectors leads to a change in the effect of PALA on the association-dissociation equilibrium.

Allosteric interactions in oligomeric proteins are ubiquitous

* This investigation was supported by United States Public Health Services Research Grant GM 12159 from the National Institute of General Medical Sciences and National Science Foundation Research Grant DMB 85-02131. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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and may play crucial roles in many metabolic regulatory circuits. The molecular mechanisms by which these interactions occur are therefore of considerable interest and have been the focus of intensive experimental and theoretical investigation. Ultimately, a satisfactory understanding of an allosteric interaction relies on a description of both the structural and energetic aspects of the communication between sites. The cooperative oxyenation of hemoglobin is an example of a particularly well characterized allosteric process, for which investigators have provided at least a partial account of the free energy of binding successive ligand molecules and have described in detail the accompanying structural changes. Allosteric interactions in a protein may be manifested in properties other than, or in addition to, ligand binding. In particular, many enzymes display allosteric behavior in steady state kinetics, either a sigmoidal dependence of velocity on substrate concentration (homotropic effects) or sensitivity to a non-substrate ligand binding at a regulatory site (heterotropic effects). Unfortunately, considerable uncertainty attends the interpretation of steady state kinetics in terms of ligand binding. In order to avoid these complications, the binding of the bisubstrate analog N-phosphonacetyl-L-aspartate (PALA) to the allosteric enzyme aspartate transcarbamoylase (ATCase, carbamoylphosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) from Escherichia coli has been investigated.

The regulatory enzyme ATCase catalyzes the first committed step in pyrimidine biosynthesis, the condensation of L-aspartate and carbamoyl phosphate to form N-carbamoyl-L-aspartate and -phosphate. The enzyme exhibits cooperativity with respect to both substrates, inhibition by CTP, and activation by ATP (1, 2). The holoenzyme, composed of two catalytic (C) trimers and three regulatory (R) dimers, is readily dissociated into free subunits (3–7). The isolated C trimers are catalytically active but display neither homotropic nor heterotropic effects, and R subunits are inactive catalytically but contain binding sites for the nucleotide effectors (3, 7). Substantial progress has been made in describing structural aspects of the allosteric behavior of ATCase. Sedimentation velocity studies have shown that the enzyme undergoes a global conformational change when it binds carbamoyl phosphate and the aspartate analog succinate (8). A similar swelling of the enzyme occurs upon binding the bisubstrate analog PALA (9). This structural change has been investigated by a variety of other techniques including chemical reactivity (10), low angle x-ray scattering (11), and x-ray crystallography (12, 13). Although PALA is a competitive

* The abbreviations used are: PALA, N-(phosphonacetyl)-L-aspartate; ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit.
inhibitor of carboxyl phosphate binding, it causes a marked activation of ATCase when present at subsaturating concentrations (14). It has been concluded, therefore, that PALA promotes the transition of ATCase to a high affinity (or high activity) swollen conformation.

In contrast to this wealth of information on the structural alterations promoted by ligand binding, direct information on the energetic aspects of these allosteric processes is relatively scarce. Instead, studies have generally relied on uncertain interpretations of kinetic data in terms of substrate affinities. Only with certain sets of microscopic catalytic rate constants will the requisite assumption that the reaction velocity is proportional to the fractional saturation of the enzyme with substrate be valid, and this premise has not been justified for ATCase. In order to avoid these complications, an investigation of the binding of PALA was undertaken. Studies by Collins and Stark (14) have shown that ATCase has a high affinity for PALA, so equilibrium binding studies are feasible only at very low concentrations of both enzyme and ligand. Accordingly, a series of equilibrium dialysis experiments have been performed with [3H]-labeled PALA. The binding isotherms of PALA to ATCase in the presence and absence of allosteric effectors, and to the isolated C subunit, have been performed. These studies yield direct energetic information on the allosteric properties of ATCase, demonstrating that ATCase binds PALA cooperatively and that the affinity of the enzyme for PALA is increased by ATP and decreased by CTP.

MATERIALS AND METHODS

Chemicals—L-[2,3-3H]Aspartic acid (14.9 Ci/mmol) was obtained from Du Pont-New England Nuclear. PALA (cyclohexylalaninum salt) was kindly provided by Dr. Jefferson Foote. Phosphonoacetic acid was obtained from Sigma, neohydrin from K&K Laboratories, AG-50W-X8 ion exchange resin from Bio-Rad, and 3A05B scintillation mixture from Research Products International Corp.

Synthesis and Purification of [3H]PALA—[3H]PALA was synthesized from L-[2,3-3H]aspartic acid and phosphonoacetic acid as described by Kempe et al. (15). The product was modified slightly to include readjustment of the reaction mixture to approximately pH 8 by the addition of 5 M NaOH after each addition of the acid chloride. This modification led to a substantial increase in the yield of [3H]PALA. The reaction product was chromatographed over AG-50W-X8 resin to remove unreacted aspartic acid. The product was neutralized to pH 7 with Tris base and was subsequently lyophilized. Excess phosphonoacetic acid proved difficult to remove chromatographically, and a procedure exploiting the high affinity of C subunit for PALA was devised for this purpose. C subunit was added to the product to give a 100-fold molar excess of active sites relative to [3H]PALA (assuming 5 sites/C subunit), ensuring that virtually all of the [3H]PALA would be bound despite the large excess of phosphonocetate. The mixture was subsequently concentrated according to the manufacturer's instructions in an Amicon Centricon-30 Microconcentrator, which retained the enzyme and any associated ligand while removing other components of the solution. This procedure was followed by several cycles of dilution of the retentate with 2 ml of 100 mM Tris-HCl buffer, and concentration of the solution. The retentate was extracted with phenol/chloroform/isoamyl alcohol (24:24:1) and chloroform/isoamyl alcohol (24:1), and the [3H]PALA-containing aqueous phase was lyophilized. The product was redissolved and addition of C subunit to the solution was repeated, although the amount of enzyme was sufficient to provide only a 20% excess of active sites over [3H]PALA. The solution was then treated as described above with several cycles of concentration and buffer addition, and the extraction procedure was repeated. The product was lyophilized and stored at -20 °C until use. This purification procedure was designed to allow the isolation of any reaction components with affinities for C subunit significantly less than that of PALA, including unreacted phosphonoacetic acid, which has a dissociation constant of approximately 0.32 mM (16). The product was assayed for radiochemical purity by chromatography on polyethylene-imine-impregnated cellulose (Polygram CEL 300 PEI from SYBRON/Brinkmann) with 1.2 M LiCl as the solvent. Only one radioactive component was observed, which co-chromatographed with authentic PALA. Further evidence of the radiochemical purity of the [3H]PALA is described under "Results."

Preparation of ATCase and Its Subunits—ATCase was prepared by the method of Gerhart and Holasek (17) from E. coli strain TR 4363 containing plasmid pPYR38 (18). C subunit was isolated by the neohydrin-mediated dissociation of ATCase, as described by Yang et al. (19). Protein concentrations were determined using extinction coefficients (280 nm) of 0.59 cm-1 mg-1 for ATCase and 0.72 cm-1 mg-1 for C subunit (17).

Equilibrium Dialysis Experiments—All experiments were performed in 40 mM potassium phosphate, 0.2 mM EDTA, 2 mM 2-mercaptoethanol at pH 7.0 (phosphate buffer). A stock solution of unlabeled PALA in distilled deionized water was prepared, and its concentration was determined by phosphate analysis (20). Low specific activity [3H]PALA solutions were prepared by the addition of [3H]PALA to dilutions of unlabeled PALA in phosphate buffer to give a final concentration of 0.02 μCi of [3H]PALA/ml. Proteins were diazoyed into phosphate buffer and were further diluted with buffer just prior to use to concentrations of 167 nM for ATCase and 333 nM for C subunit. When nucleotides were present, the final concentrations were 2 mM for ATP and 0.5 mM for CTP, and equimolar magnesium acetate was included. Dialysis membrane (Spectra/Port 4, molecular weight cutoff 12,000-14,000, Fisher Scientific) was rinsed several times in 2 mM EDTA and was stored in 0.2 mM EDTA at 4 °C prior to use. Dialysis was performed in cylindrical tubes cells constructed according to the design of Myer and Schellman (21), with a radius of 1 cm and a total volume of 0.8 ml. A [3H]PALA solution (0.35 ml) was loaded into one half-cell and an enzyme (or enzyme/nucleotide) solution (0.35 ml) into the other, and the cells were placed on a rocking platform at 23 °C for approximately 8 h prior to removal and analysis of the solutions. Control experiments showed effective equilibration in less than 6 h under these conditions, in the presence or absence of protein (data not shown). Scintillation mixture (4 ml) was added to three separate 0.10-ml aliquots from each chamber, and radioactivity measurements were performed in a Beckman LS-7500 scintillation counter.

Data Analysis—Average dissociation constants, Kd (identical with Wyman's "median ligand activity" (22)), for the cooperative holoenzyme were estimated by nonlinear least-squares analyses of the data sets in terms of the allosteric model of Monod, Wyman and Changeux (23). The saturation function of the model was reformulated, as suggested by the analysis of Wyman (24), to include a factor corresponding to (Kd)6, as well as the dissociation constant of the ligand from the T state, Kd, the ratio of the concentrations of the T and R states of the unligated enzyme, L, and the fractional maximal saturation corresponding to a given [3H]PALA concentration. Confidence intervals on Kd were determined by F tests and correspond to 1 S.D. (25). Hill coefficients (nH) and the free ligand concentrations of the T and R states of the unligated enzyme, L, and the fractional maximal saturation corresponding to a given [3H]PALA concentration were calculated from the resulting parameter sets. The holoenzyme data sets were also fit to the standard Monod-Wyman-Changeux saturation function by nonlinear least squares analysis with some parameters constrained, as described under "Discussion." Data for the C subunit were fit to a hyperbola by nonlinear least squares analysis, and confidence limits corresponding to 1 S.D. were determined by the method of Johnson and Frazer (26).

RESULTS

PALA Binding to ATCase—The binding isotherms for PALA to ATCase in the absence of nucleotides, in the presence of 2 mM ATP, and in the presence of 0.5 mM CTP are shown in Fig. 1a. The concentration of free ligand at half-saturation of the enzyme, Kd, and the average dissociation constant, Kd, were 98 nM and 110 nM, respectively, in the absence of effectors. In the presence of ATP, Kd, and Kd, decreased to 53 nM and 65 nM, respectively, and increased to

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1 Experiments were performed in phosphate buffer since the data in non-phosphate buffers was less reproducible. Additionally, phosphate buffer has been used in numerous other studies, so direct comparison of the results is warranted. Since phosphate buffers to the active site of the enzyme, the apparent dissociation constant determined in the absence of phosphate would be several-fold lower.
Fig. 1. PALA-binding isotherms of ATCase in the presence and absence of allosteric effectors. Equilibrium dialysis experiments were performed as described under "Materials and Methods" at 23 °C in 40 mM potassium phosphate buffer containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol at pH 7.0. Where indicated, ATP and CTP were at concentrations of 2.0 mM and 0.5 mM, respectively. The enzyme concentration was 167 nM. Data in the absence of effectors are designated by O, in the presence of ATP by Δ, and in the presence of CTP by Ο. The solid lines correspond to the best fits of the data sets described under "Materials and Methods." a shows the data plotted as [PALA]bound/[ATCase] versus [PALA]free. b shows the data plotted as ([PALA]bound/[ATCase])/[PALA]free versus [PALA]bound/[ATCase].

216 nM and 266 nM in the presence of CTP. The binding of PALA is cooperative both in the presence and absence of nucleotides, as is evident in the Scatchard plot of the data (Fig. 1b), on which an equation describing binding to identical, noninteracting sites follows a straight line, while positive cooperativity in binding generates a curve which is concave with respect to the abscissa. The value of nH is 1.95 in the absence of nucleotides, 1.35 in the presence of ATP, and 2.27 in the presence of CTP. The maximal fractional saturation corresponds in each case to between 5.4 and 5.7 sites per enzyme molecule, within 10% of the expected six sites. These results are summarized in Table I.

PALA Binding to Isolated C Subunits—The binding isotherm of PALA to the isolated C subunit of ATCase is presented in Fig. 2a and as a Scatchard plot in Fig. 2b. Analysis of the data in terms of a hyperbola gives 2.34 binding sites with a dissociation constant of 98 nM. This value for the number of binding sites is clearly inconsistent with structural information, which indicates the existence of three active sites/C subunit. Different preparations of C subunit also demonstrated this apparent deficiency of sites, although the maximal saturation observed was more variable than that observed with different holoenzyme preparations suggesting that the binding site deficiency might be due to damage incurred during the preparation of C subunit. This possibility was investigated by adding an excess of regulatory subunits to a preparation of C subunit and determining the apparent number of PALA binding sites of the reconstituted holoenzyme. The reconstituted ATCase showed only 4.7 binding sites/enzyme molecule, consistent with the view that C subunit had been damaged during the isolation procedure with a resultant loss of an average of 0.4 binding sites/trimer. PALA binding to these sites appears not to be totally precluded, however, since experiments at high concentrations of C subunit (3 μM) saturate at a higher number of sites (about 2.7/trimer, data not shown), indicating the presence of a class of sites with a much lower affinity for PALA.

There is an obvious deviation of the data from the C subunit at low saturation from the best fit line, most apparent in the Scatchard plot of the data (Fig. 2b). This pattern indicates the existence of a slight degree of positive cooperativity in PALA binding by the C subunit. An alternative explanation for this deviation, the presence of a radiolabeled impurity binding less tightly than PALA, seems unlikely, considering

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### Table I

| Parameters for PALA binding to ATCase and to the isolated C subunit |
|-----------------|-----------------|-----------------|-----------------|
|                 | ATCase          | C subunit       |
| K<sub>a</sub> or K<sub>a</sub> (nM)<sup>a</sup> | 98              | 216             | 95              |
| K<sub>H</sub> (nM)      | 110             | 266             |                 |
| n<sub>H</sub>       | 1.95            | 1.35            | 2.27            |
| Sites<sup>b</sup> | 5.45            | 5.54            | 5.68            |
|                   | 284             | 216             | 234             |

<sup>a</sup> K<sub>a</sub> is reported for ATCase. K<sub>a</sub> is reported for C subunit; confidence limits are approximately 74 and 115 nM.

<sup>b</sup> The confidence limits on K<sub>H</sub> are 107 and 120 nM in the absence of nucleotides, 64 and 73 nM in the presence of ATP, and 246 and 284 nM in the presence of CTP.

<sup>c</sup> The calculated average maximal saturation. Errors are approximately ±0.1 in each case.
the specificity of the procedure used for purifying the [3H]
PALA. Furthermore, ligand heterogeneity of this type would
give rise to different binding isotherms as the protein concen-
tration is varied, but binding experiments at dramatically
different C subunit concentrations (0.05 to 3 μM) gave similar
isotherms (data not shown).

DISCUSSION

An allosteric interaction may be considered the effect the
binding of a ligand to one site on a macromolecule has on the
properties of another site. These interactions are thought to
generally involve utilization of the ligand binding energy to
drive a conformational isomerization in the macromolecule.
Many studies of allosteric systems focus on elucidating the
mechanism by which ligand binding is coupled to equilibria
between conformational states of the macromolecule, and
various models of the linkage between ligand binding and
conformational isomerizations for an allosteric macromole-
cule have been advanced. Any evaluation of these models
must be based on both structural and energetic information.
Often allosteric enzymes are analyzed in the absence of any
direct information about ligand binding by assuming that the
steady state reaction velocity is proportional to the fractional
saturation of the enzyme with substrate. There are, however,
many possible kinetic schemes in which this assumption
would be invalid and could lead to incorrect conclusions,
including the existence of multiple kinetic pathways, nonpro-
ductive modes of substrate binding, or a dependence of any
microscopic rate constants on the fractional saturation.
A direct investigation of ligand binding is therefore crucial to a
reliable interpretation of the properties of an allosteric en-
zyme.

ATCase is a textbook example of an allosteric enzyme,
exhibiting both homotropic and heterotropic effects (27).
Some structural aspects of the allosteric behavior of ATCase
are relatively well characterized. The enzyme undergoes a
large conformational change in the presence of certain ligands
which may be accurately monitored by velocity sedimentation
(8) and which has been investigated at the atomic level by x-
ray crystallography (12, 13). However, the application of
thermodynamic models of allosteroy to ATCase has been com-
licated by the lack of any direct information on the binding
of a ligand which promotes this characteristic conformational
change in the enzyme and has instead usually relied on
uncertain interpretations of kinetic data. In this study, these
complications have been circumvented by investigating the
binding of the bissubstrate analog PALA to ATCase, in the
presence and absence of allosteric effectors, and to the isolated
C subunit of the enzyme.

Since PALA promotes the allosteric transition of ATCase,
it was expected that PALA would bind cooperatively to the
enzyme. As shown in Fig. 1, PALA binds to ATCase with
marked cooperativity, which is especially apparent in the
curvature of the Scatchard plot of the data (Fig. 1b). The Hill
coefficient at half-saturation ($n_H$) is commonly used as a
phenomenological index of the degree of cooperativity. The
value of $n_H$ for PALA binding in the absence of either nucleo-
tide is 1.95, intermediate between no cooperativity ($n_H = 1$)
and complete cooperativity (for which $n_H$ would equal the
number of binding sites, 6 in this case). The effects of ATP
and CTP on the cooperativity of PALA binding are qualita-
tively analogous to their effects on the kinetic cooperativity
of the enzyme; ATP decreases the cooperativity of PALA
binding ($n_H = 1.35$), while CTP increases it ($n_H = 2.27$).
In addition to altering the kinetic cooperativity exhibited by
ATCase, the allosteric effectors ATP and CTP alter the
concentration of the substrate aspartate required for half-
maximal velocity, which is decreased by ATP and increased
by CTP. These changes have been interpreted in terms of
alterations in the affinity of the enzyme for aspartate. ATP
and CTP might, therefore, also be expected to alter the
affinity of the enzyme for PALA. The data confirm this
prediction. As shown in Table I, the average dissociation
constant ($K_{av}$) of PALA from ATCase is decreased from 110
nM to 65 nM by ATP and is increased to 266 nM by CTP.
From the relationship between $K_{av}$ and the free energy for
saturating a macromolecule with a ligand, $\Delta G_{0,av}$, which equals
$-RT\ln K_{av}$ (where $t$ is the number of binding sites), it is
calculated that saturation of the six binding sites of ATCase
with PALA is 1.9 kcal/mol more favorable in the presence of
ATP than in its absence and 3.1 kcal/mol less favorable in
the presence of CTP than in its absence.

The binding of PALA to the isolated C subunit was also ex-
amined. The C subunit is composed of three identical poly-
peptide chains with C$_{3}$ symmetry, suggesting the presence
of three active sites (12). As described under "Results," C
subunit prepared by neohydrin-mediated dissociation of the
holoenzyme exhibits less than the expected number of binding
sites, approximately 2.3/trimer. Similar results have been
described for PALA inhibition of the arsenolysis of N-carba-
myl-L-aspartate catalyzed by the C subunit (28). Reconsti-
tution experiments also described under "Results" suggest
that this binding site deficiency is the result of damage to a
subset of binding sites incurred during the isolation of the C
subunit, with a dramatic decrease in the affinity of these sites
for PALA. The remainder of this discussion will be based on
this explanation of the unusual binding stoichiometry, al-
though alternative mechanisms have not been ruled out. Since
the C subunit of ATCase shows no cooperativity in enzyme
kinetics with respect to either aspartate or carbamoyl phos-
phate, it seemed likely that PALA would bind noncooper-
avely to the C subunit. In fact, PALA binds to the C subunit
with a very slight degree of cooperativity as indicated by the
slight deviation from linearity in the Scatchard plot of the
data (Fig. 2b). Complex saturation curves have previously
been reported for the binding of carbamoyl phosphate to the
isolated C trimer (29). The conformational change presumed
to underlie this cooperativity may have been previously ob-
served in experiments described by Lahue and Schachman
(30), which demonstrate that the binding of PALA to one site
on a C subunit causes a perturbation of the absorption spec-
trum of a chromophore at an unligated site on the same C
subunit (30). The best fit of the binding data for the C subunit
to a hyperbola corresponds to a dissociation constant of 95
nM.

Ligand binding and assembly of subunits into oligomers are
coupled energetically (31) and provide complementary infor-
mation for allosteric systems. Consideration of the thermo-
dynamic linkage between assembly of ATCase from isolated
C and R subunits and the binding of the ligands ATP, CTP,
and PALA permits some conclusions about the energetics of
subunit interactions to be drawn from the PALA-binding data.
A linkage scheme relating these two sets of reactions is
shown below:

\[ \begin{align*}
2\text{C} + 3\text{R} + 6\text{PALA} & \rightarrow 2\text{C-PALA} + 3\text{R} \\
\Delta G_{1} & = \Delta G_{\text{C-PALA}} \\
\Delta G_{2} & = \Delta G_{\text{C-PALA}} \\
\text{C$_{2}$R$_{3}$ + 6PALA} & \rightarrow \text{C$_{2}$R$_{3}$-PALA} \\
\Delta G_{3} & = \Delta G_{\text{C$_{2}$R$_{3}$-PALA}}
\end{align*} \]
The PALA-binding reactions of the holoenzyme, with a free energy change designated $\Delta G_{\text{holo}}$, and of the two C subunits from which it is assembled, corresponding to $2\Delta G_{\text{cat}}$, are represented by the horizontal segments of the diagram. These quantities are calculated from the binding data as 56.7 and 2(28.6) kcal/mol, respectively.\textsuperscript{3} The difference between these two segments has been termed the cooperative free energy and is a measure of the free energy expended in order to regulate the ligand binding process (32). The calculated cooperative free energy for PALA-binding by ATCase is small, about 0.5 kcal/mol of holoenzyme. The vertical segments represent the subunit association-dissociation reactions in the absence and presence of PALA, with free energy changes designated $\Delta G_s$ and $\Delta G_{\text{PALA}}$, respectively. Since the sum of these quantities is equal to the cooperative free energy, the binding of 6 molecules of PALA to ATCase shifts the association-dissociation equilibrium 0.5 kcal/mol toward the free subunits. PALA-promoted dissociation of a form of ATCase in which one of the regulatory subunits is missing has, in fact, been detected in sensitive subunit exchange experiments, described by Subramani and Schachman (33).\textsuperscript{3} The PALA-binding data also demonstrate that the effect of PALA on the association-dissociation equilibrium is altered when the R subunits are complexed with either allosteric effector. In the presence of ATP, the magnitude of the PALA-promoted shift toward dissociation is increased from 0.5 to 3.7 kcal/mol. In the presence of PALA, the binding of PALA actually favors association of the subunits into holoenzyme by 1.3 kcal/mol.

The energetics of PALA binding has been the subject of previous investigations. From analysis of the inhibition of the steady state reaction by PALA (34, 35), McCarthy and Allewell (36) have given the cooperative free energy as 3.6 kcal/mol of holoenzyme, substantially greater than the 0.5 kcal/mol reported here. The difference between these values might be due to differences in experimental conditions or to assumptions used in analyzing those experiments. The enthalpy of PALA binding to ATCase and to the C subunit have been measured by Knier and Allewell (35) and by Shrake and Schachman (37). The conclusions of those studies can be confirmed by determining the effect of temperature on the PALA-binding isotherms.

A satisfactory understanding of allosteric effects requires not only a description of the energetics of the interactions between ligands, but also of the molecular mechanisms by which they occur. Of the various theories formulated to correlate the thermodynamic and structural aspects of allosteric interactions, the two-state (or concerted) model of Monod, Wyman and Changeux (23) has proven most useful in describing ATCase. In the two-state model, the allosteric macromolecule exists in an equilibrium between two distinct conformational isomers, the T and R states, which may differ in their affinities for various ligands. Homotropic and heterotropic effects are the result of ligand-promoted shifts in this conformational equilibrium. The description of a system within the mathematical framework of this model requires a value for the ratio of the concentrations of the T and R states of the unligated macromolecule (this quantity is the allosteric equilibrium constant, $L_0$) and the affinity of each state for every ligand being considered. Since the results of many experimental investigations of ATCase may be accounted for by the two-state model with a single set of parameters (10), it was of interest to determine whether the PALA-binding data could be described by the model using these previously determined constraints. Values of $L_0$, $c_{\text{PALA}}$, the ratio of the dissociation constants of PALA from R and T states of ATCase, have been given as 250 and 0.028, respectively, under the conditions used in the present studies (10). Fixing these parameters and varying only the affinity of PALA for the R state of the enzyme, $K_R$, which had not been previously determined, the best fit of the PALA-binding isotherm in the absence of nucleotides is obtained with $K_R$ equal to 45 nM. This analysis indicates that the affinity of the R-state enzyme for PALA is greater than that of the C subunit. In the original description of the two-state model (23), it was supposed that each of the two quaternary states would be "constrained" with respect to the free subunits and would subsequently have a lower affinity for the ligand, which the present analysis indicates is not the case for PALA binding to ATCase. This concept of constraint, while providing one structural explanation for the origin of the cooperative free energy, is not a factor in the description of the behavior of an assembled allosteric macromolecule provided by the two-state model. The role of the allosteric effectors in the model would be to alter the position of the equilibrium between the two quaternary states while leaving the affinity of each state for the ligand unchanged. Accordingly, the binding isotherms in the presence of nucleotides were fit by fixing $c_{\text{PALA}}$ to the previously determined value of 0.028 and $K_R$ to the value derived from the isotherm in the absence of nucleotides, 45 nM, varying only the value of the allosteric equilibrium constant for the effector-ligated enzyme, $L_{\text{ATP}}$, or $L_{\text{CTP}}$. The best fits were obtained with $L_{\text{ATP}}$ equal to 7.2 and $L_{\text{CTP}}$ equal to 19,000.\textsuperscript{4}

In each of these analyses, the resulting set of parameters provided a reasonable description of the data, although in each case statistically better fits could be obtained by relaxing other parameter constraints.

These studies demonstrate the feasibility of measuring PALA binding by equilibrium dialysis and illustrate some of the advantages of this approach, principally the ability to obtain sound thermodynamic information about this allosteric system. The introduction of structural perturbations in a protein, recently via site-directed mutagenesis, is a valuable technique for probing the energetic and structural aspects of allosteric interactions. Indeed, mutational studies of ATCase have yielded insights into the allosteric properties of the enzyme (38-42). The results of the experiments described here generally support the conclusions derived from interpreting steady state kinetic data in terms of substrate binding. Nevertheless, given the variety of effects which a mutation may have on the structure and function of an enzyme, deriving conclusions about the allosteric properties of an enzyme through a comparison of the catalytic properties of mutant enzymes is hazardous. Analysis of the simpler process of PALA binding, on the other hand, should permit an accurate assessment of the effects of mutational alterations on the energetics of allosteric interactions. An additional advantage

\textsuperscript{3} The value for the C subunit was calculated from the best fit of the data to an equation describing identical and independent sites; the effect of the exhibited degree of cooperativity on this value is negligible.

\textsuperscript{4} The difference between these values and those reported by Howlett et al. (10) may be attributed to the use of magnesium salts of the nucleotides in this study, which have previously been shown to produce more pronounced heterotropic effects. In addition, uncomplexed magnesium ions may slightly weaken PALA binding to ATCase since these ions have been shown to inhibit enzyme activity (43).
is the range over which the affinity of the enzyme for PALA may vary while still permitting determination of a useful binding isotherm. Preliminary results suggest that a decrease in affinity of greater than 3 orders of magnitude should not significantly decrease the quality of the PALA-binding isotherm. In contrast, a 50-fold increase in the $K_D$ of the enzyme for aspartate, for example, would preclude the determination of a useful kinetic saturation curve.

Acknowledgment—We appreciate the assistance of Dr. Charles H. Robert in analyzing the data.

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