BINDING OF CYTIDINE TRIPHOSPHATE TO ASPARTATE TRANSCARBAMYLASE

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SUMMARY

Equilibrium dialysis studies reveal that cytidine triphosphate (CTP) binds to a total of six sites on native aspartate transcarbamylase (ATCase) from *E. coli*. Analysis of the binding curves suggests that there are two discrete classes of CTP binding sites on each native ATCase molecule. Two models are discussed in which a paired arrangement of regulatory chains in the native enzyme leads to heterogeneous binding of CTP. One model assumes that the folding of polypeptide chains in the regulatory dimer produces two CTP binding sites which are physically different and differ in their intrinsic affinities for CTP. Alternatively, interference between CTP molecules binding to identical CTP sites on each regulatory dimer may cause the two sites to bind the ligand with different affinities.

Studies by Changeux *et al.* (1) on the binding of ligands to aspartate transcarbamylase from *E. coli* appeared to indicate the existence of four specific sites for succinate (an analog of one of the substrates, aspartate) and four specific sites for the inhibitor CTP, per molecule of enzyme. Combination of these data with evidence from studies on subunit composition, (10) symmetry elements occurring in crystalline ATCase (16), and polypeptide composition of the isolated subunits (11,17) led to proposals that ATCase is a tetrameric molecule, composed of two catalytic subunits (each containing two polypeptide chains and two succinate binding sites) and four regulatory subunits (each with one chain and one CTP binding site). However, Weber (18) has subsequently presented evidence, derived from studies of amino acid sequence in the regulatory chain and from the rates of migration of denatured subunits in polyacrylamide gels, indicating that the subunit molecular weights are too low to be consistent with a tetrameric structure of ATCase. He proposed a model in which the enzyme contains six of each kind of polypeptide chain. The latter model has received support from further X-ray crystallographic work (19), studies of electrophoretic migration of ATCase "hybrids" reconstituted from succinylated and nonsuccinylated subunits (13), and studies on crosslinking of polypeptide chains in ATCase (4). Studies on the structure of ATCase have recently been reviewed in some detail by Gerhart (8).

As pointed out by Weber (18), most of the older data can be reinterpreted to fit the hexameric structure for ATCase but the ligand binding data of Changeux *et al.* (1) stand out in apparent contradiction to the newer model for the structure of the enzyme. In the studies reported below, we have begun a reinvestigation of the ligand binding behavior of ATCase, measuring the binding of the inhibitor CTP.

METHODS AND MATERIALS

Equilibrium dialysis experiments were conducted in Lucite microdialysis cells (7) using Visking 20/32 dialysis tubings (Union Carbide) which had been stretched to increase their porosity (3). Solutions were added to or withdrawn from the dialysis chambers through finely drawn-out glass capillary tubes; volumes of samples removed from the chambers were measured by transfer to microcapillary pipettes (Drummond "Microcaps", Kensington Scientific). This procedure permitted measurement of aliquots of 10 or 15 microliters with a reproducibility of ± 1%. Native ATCase from *Escherichia coli* was prepared as described by Gerhart and Holoubek (9). 2-^{14}C-CTP was purchased from Schwarz and was purified on a Dowex-1-bicarbonate column prior to use. Protein concentrations in the dialysis chambers were determined at the conclusion of each experiment by the Lowry method (12) using as the standard a solution of ATCase whose concentration had been determined by the synthetic-boundary method of Schachman (15).

RESULTS

Data from a typical equilibrium dialysis experiment are shown in Figure 1. Binding of CTP to ATCase is expressed in terms of $\bar{\nu}$, the number of molecules of CTP bound per molecule of enzyme; [CTP] is the molar concentration of unbound CTP. The data are presented in the form of a Scatchard plot (14). A linear plot would indicate that there is a single class of equivalent and independent CTP binding sites on the enzyme. These data exhibit a marked deviation from linearity, suggesting the existence of more than one class of sites. It was found that the data could be fitted very well by assuming that there are two classes of equivalent CTP binding sites on ATCase, each class containing 2.9 sites, with the association constant for the high-affinity class, K_1, equal to 1.1×10^6 $\underline{M}^{-1}$ and that for the low-affinity class, K_2, 2.5×10^4 $\underline{M}^{-1}$. The solid black line seen in the left-hand portion of the figure is the reconstructed Scatchard plot derived by using these assumed values in the equation

$$\bar{\nu}_{total} = \bar{\nu}_1 + \bar{\nu}_2 = \frac{n_1 K_1 [CTP]}{1 + K_1 [CTP]} + \frac{n_2 K_2 [CTP]}{1 + K_2 [CTP]}$$

and solving for $\bar{\nu}_{total}$ at a variety of concentrations of free CTP. The dashed lines in the graph represent the contributions of the individual

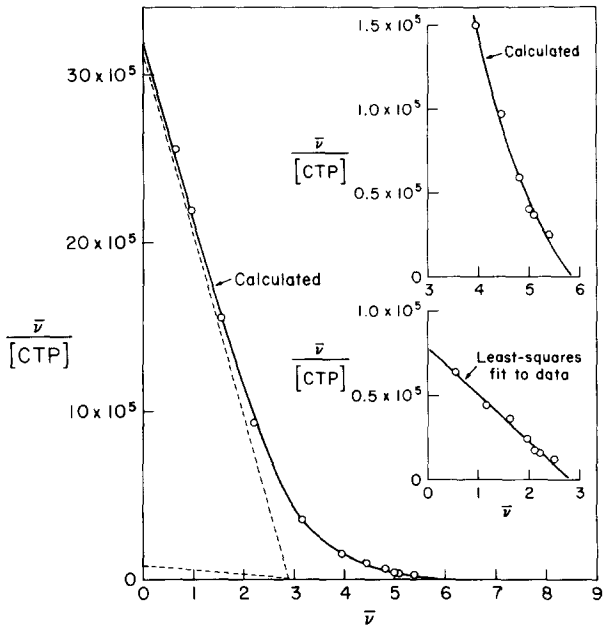

Figure 1. Binding of CTP to native ATCase. Equilibrium dialysis was carried out at 4°C in 0.01 M potassium phosphate pH 7.0, 0.002 M 2-mercaptoethanol, and 0.0002 M sodium EDTA. ATCase was present at 20 mg/ml; free CTP at 2 x 10^{-7} to 2 x 10^{-4} M. Dialysis time was 24 hours. See text for definition of terms and analysis of data. No correction has been made for Donnan effects; however, controls in which dialysis was carried out in the presence of 0.1 M KCl gave essentially the same results as were obtained in the absence of added salt.

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classes of binding sites to the reconstructed plot ($\bar{\nu}_1/[CTP]$ and $\bar{\nu}_2/[CTP]$, plotted separately as a function of concentration of free CTP).

The total number of CTP binding sites obtained by plotting the reconstruction giving the best fit to the data is n = 5.8. The extrapolation to 5.8 sites per enzyme is shown clearly in the upper insert in Figure 1, in which the lower portion of the Scatchard plot is expanded. It is seen that the data are fitted very closely by the reconstructed curve at all values of $\bar{\nu}$ observed.

The lower insert in Figure 1 represents a test of the assumption of two classes of binding sites, carried out as follows. The existence of a class of 2.9 high-affinity sites with K_1 = 1.1 x 10^6 M^{-1} was assumed, and for each experimental CTP concentration the $\bar{\nu}$ for this class of sites was calculated from the relation (6) $\bar{\nu}_1 = n_1 K_1 [CTP]/1 + K_1 [CTP]$. The calculated $\bar{\nu}_1$ was then subtracted from the total $\bar{\nu}$ observed at each CTP concentration; a Scatchard plot of the resulting data is shown in the lower insert. If the low-affinity

binding sites constitute a single class of equivalent sites, these points should fall on a straight line. Within the limits of experimental error, this appears to be the case; further, a least-squares fit through the points yields a number of sites (2.81 ± 0.06) consistent with the extrapolated total of 5.8 CTP sites per enzyme molecule.

The results described above were confirmed in two additional equilibrium dialysis experiments carried out under conditions essentially identical to those described in Figure 1. Binding constants derived from the three experiments are compared in Table 1; variations in the value of n are attributed to uncertainty (± 5%) in the determinations of protein concentration for each experiment. Further studies on the binding of CTP to native ATCase were conducted using a novel method which we have developed for the rapid determination of ligand-protein binding affinities (partition equilibrium method (20)). These studies involved determination of the effect of ATCase on the distribution of CTP in a biphasic aqueous system containing the polymers Dextran and polyethylene glycol. The partition equilibrium experiments (20) yielded a non-linear Scatchard plot which can be fitted by assuming the existence of two classes of CTP binding sites on ATCase, in agreement with the equilibrium dialysis results described in this paper. The total number of CTP binding sites per ATCase molecule observed by the method of partition equilibrium was n = 6.1.

Experiment	K_1	K_2	n
1	1.0×10^6	2.0×10^4	6.3
2	0.9×10^6	1.7×10^4	5.8
3	1.1×10^6	2.5×10^4	5.8
Average	1.0×10^6	2.1×10^4	6.0

Table 1. Binding constants derived from equilibrium dialysis experiments with native ATCase. See text for a description of the methods used to derive the binding constants.

DISCUSSION

The results presented above demonstrate that there are six binding sites for CTP on each molecule of native ATCase. The binding of CTP to ATCase is not homogeneous; at high concentrations of CTP, the affinity of that ligand for remaining CTP sites on the enzyme is reduced compared to its affinity at

low CTP concentrations. Analyses of Scatchard plots of the binding data suggest that there are two discrete classes of CTP binding sites on each ATCase molecule. Thus the data are closely fitted by theoretical curves which assume two classes of independent and equivalent binding sites on ATCase, each class containing three sites.

How can one reconcile our results with the subunit structure of ATCase? We assume that native ATCase contains six identical regulatory polypeptide chains, each of molecular weight 17,000 (18). The isolated "regulatory subunit" of Gerhart and Schachman (10) is probably a dimer of regulatory polypeptide chains (18), since it has a molecular weight estimated at 30,000 (10). Studies of the crosslinking of polypeptide chains in native ATCase suggest that pairs of regulatory chains are arranged in close proximity to each other in the native enzyme molecule (4,5). Thus it seems likely that regulatory polypeptide chains in native ATCase are arranged in pairs, which we will term regulatory dimers. Changeux *et al.* (1) have studied the binding of CTP to isolated "regulatory subunits" and have observed two CTP binding sites, differing roughly twentyfold in their affinity constants. If the isolated "regulatory subunit" is actually a dimer of regulatory chains, then heterogeneous binding of CTP to such a regulatory dimer probably reflects nonequivalent binding of that ligand to the two polypeptide chains composing the dimer. These observations, when taken with our own results, suggest that heterogeneity in the binding of CTP to native ATCase is a consequence of heterogeneous binding of CTP to each of the regulatory chains in a regulatory dimer.

There are two ways in which nonequivalent binding of CTP to two sites on a regulatory dimer could occur. First, the geometric arrangement of the two polypeptide chains composing each dimer might be asymmetric with the consequence that the two CTP binding sites are physically different and differ in their intrinsic affinities for CTP. Each regulatory dimer would then bear a high-affinity and a low-affinity CTP binding site. If each molecule of ATCase contains three regulatory dimers, this would give rise to three sites in each of two classes of CTP binding sites. Alternatively, one might assume that the two CTP sites on each regulatory dimer are identical so that ATCase contains six equivalent binding sites for CTP. If this is true then the appearance of two classes of CTP binding sites on ATCase must be due to interactions between CTP binding sites. One such possibility would envisage that the binding of CTP to one site in a regulatory dimer reduces the affinity of the other site for a second molecule of CTP. Such negative interactions could result from direct electrostatic or steric interference between the two CTP molecules, or indirectly from conformational changes in the regulatory subunit brought about by binding of the first CTP. Our data do not indicate

whether heterogeneous binding of CTP to ATCase is due to ligand interactions or to intrinsic differences among the CTP binding sites. We are presently testing the binding affinities of a variety of ligands for all six CTP sites in an attempt to characterize intrinsic differences among the sites, if such differences exist.

A number of apparent inconsistencies are resolved by the finding that there is a heterogeneity of CTP binding sites on native ATCase. Changeux *et al.* (1) reported four CTP binding sites per molecule of native ATCase, but in fact these authors did see some indications of a second class of low-affinity sites (see especially Changeux and Rubin, 1968, Figure 8). It seems likely that the data used for extrapolation to an apparent n = 3.8 sites (Changeux *et al.*, 1968, Figure 2) represent only the first three of a total of six sites. If this is the case, it is expected that the extrapolated value of n for the first class of sites (the high-affinity sites) will be too high; direct linear extrapolations of portions of the Scatchard plot cannot be applied in cases where there is more than one class of binding sites for a given ligand. The apparently homogeneous BrCTP binding observed in ultracentrifuge experiments by Gerhart and Schachman (1965) may be a consequence of the fact that their binding data were obtained at $\bar{\nu} \geq 3$, so that no information was obtained on the binding affinity of the high-affinity sites.

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