From allostery to mutagenesis: 20 years with aspartate transcarbamoylase

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Following the discovery by Gerhart & Pardee (1962) that aspartate transcarbamoylase (ATCase, EC 2.1.3.2) from Escherichia coli is inhibited by CTP and that there is a sigmoidal dependence of activity on aspartate concentration, much effort has been focused on ATCase as an allosteric enzyme. In particular, research has been aimed at accounting for both the heterotropic effects (inhibition by CTP and activation by ATP) and the homotropic effects (cooperativity with respect to substrate concentration) in terms of molecular models. Considerable success has been achieved in interpreting many diverse experimental findings (Howlett et al., 1977) according to the two-state model proposed by Monod et al. (1965) and this communication summarizes further studies aimed at testing the validity of that model. The availability of altered forms of ATCase produced by site-directed mutagenesis provides an opportunity to evaluate the effects of structural changes on some of the parameters describing the allosteric transition and to probe the nature of the active sites within the enzyme. Some recent results are presented here in outline form.

ATCase as an allosteric enzyme

There is general agreement that the sigmoidal dependence of enzyme activity on substrate concentration is attributable to a substrate-promoted conformational change in the enzyme whereby it is converted from a low-activity T-state to a conformation of higher activity (R-state). A conformational change in ATCase was demonstrated unequivocally by ultracentrifuge studies which showed that the sedimentation coefficient decreased by about 3% upon the binding of substrate analogues (Gerhart & Schachman, 1968). Since a decrease in sedimentation coefficient upon ligand binding could be interpreted only as a swelling of the enzyme or a change in its shape to a more anisometric form, we adopted about 15 years ago the model shown in Fig. 1 for the T- and R-conformations. Subsequent crystallographic studies (Krause et al., 1985), which have provided much more precise evidence about the conformation of the six catalytic and six regulatory chains in the holoenzyme, have indeed confirmed that there is a large change in quaternary structure and that the two catalytic trimers within ATCase are further apart in the R-state than in the T-state. These studies showed also that there is a rotation of each of the trimers about the 3-fold axis when the enzyme binds the bisubstrate ligand, N-(phosphonacetyl)-L-aspartate (PALA).

It should be recognized that all interpretations of sigmoidal enzyme kinetics in terms of models have been based solely on changes in the affinity of the enzyme for aspartate, and no consideration was given to possible changes in the catalytic activity as the enzyme was converted from the T- to the R-state. A reliable value for $V_{\text{max}}$ of the R-state enzyme is readily obtainable experimentally but no corresponding determination of the value for the native T-state enzyme is feasible because of the substrate-promoted conformational change. Therefore, there is considerable risk in the use of enzyme kinetics alone in testing the validity of the two-state model. In this regard a promising beginning has been made in circumventing this difficulty by direct binding studies with the bisubstrate ligand PALA (D. W. Markby, J. Newell & H. K. Schachman, unpublished work). By doing equilibrium dialysis experiments with radioactive PALA we have been able to demonstrate co-operativity in its binding to ATCase; in addition, there is a shift in the sigmoidal saturation curve to higher PALA concentrations when CTP is present and a corresponding shift to lower concentrations upon the addition of ATP. Control experiments with the isolated catalytic (C) trimers which exhibit Michaelian kinetics yield hyperbolic binding curves with radioactive PALA. As yet a precise evaluation of the allosteric parameters is not available, but the method shows sufficient promise that tests of models in the same manner that has

Abbreviations used: ATCase, aspartate transcarbamoylase; PALA, N-(phosphonacetyl)-L-aspartate; C, catalytic subunit; R, regulatory subunit; N, as subscript, native; NIT, as subscript, nitrated; P, as subscript, pyridoxylated; DNP, as subscript, dinitrophenyl.

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The lower catalytic subunit, C\textsubscript{NT,F}, contains a sensitive chromophore incorporated by nitration of about one tyrosine residue per chain and it is inactivated by pyridoxylation of lysine 84. This doubly modified subunit designated by the heavy stippling does not bind succinate even in the presence of carbamoylphosphate. The other subunit in the hybrid is native and binds succinate as indicated by the light stippling on the right. The hybrid exhibits both homotropic and heterotropic effects. Below the model is the difference spectrum caused by the binding of succinate (Yang & Schachman, 1980).

Communication between polypeptide chains in ATCase

Because ATCase is readily dissociated into separable catalytic and regulatory subunits and the reconstitution of the holoenzyme is very efficient (Gerhart & Schachman, 1965), we have been able to incorporate sensitive chromophores into either of the two types of polypeptide chains in ATCase-like molecules. With such derivatives we have demonstrated that ligand binding to the three active catalytic chains in one trimer causes a global conformational change in a hybrid molecule in which the other three inactive catalytic chains which are linked to each other non-covalently from the catalytic and regulatory chains (C. Hu & H. K. Schachman, unpublished work). Fig. 3 shows a schematic representation of the hybrid and a difference spectrum for the binding of PALA to the derivative C\textsubscript{P}(R\textsubscript{DNP})\textsubscript{3}. Even though the chromophores on the regulatory subunit (R\textsubscript{DNP}) are at a considerable distance from the site of PALA binding in the C subunits, there is a marked alteration in the absorption spectrum of the dinitrophenyl groups which is linked to the quaternary structural change promoted by PALA binding. Analogous experiments in which the chromophore is placed on the catalytic chains show that CTP and ATP binding to the native regulatory chains promote significant and opposite changes in the absorption spectrum of the nitrotyrosyl groups.

Concerted nature of the allosteric transition

A variety of approaches have been used in our laboratory to determine whether the T → R transition is concerted or alternatively whether there is an accumulation of intermediates in which part of a molecule is in the T-state and the remainder has the R-conformation. On the basis of the unusual architecture of ATCase it is tempting to speculate, as some workers have done, that distinct parts of the molecule function as co-operative independent entities. For ATCase there would appear to be two feasible models containing such co-operative substructures. One involves a combination of two catalytic chains and two regulatory chains which are linked to each other non-covalently from one C trimer to that beneath it. In this postulated model co-operativity would be achieved largely through the mediation of the two regulatory chains within the connecting R dimer. This co-operative unit can be ruled out since hybrids (like that in Fig. 2) containing one totally inactive C subunit exhibit allosteric properties (Yang & Schachman, 1980). Therefore allostery is not dependent on catalytically func-
tional chains in both trimers. In the other model the co-operative unit is assumed to encompass two or three catalytic chains in either of the two C subunits. But this proposal is shown to be invalid by the demonstration that hybrids containing only one active catalytic chain along with two inactive chains in each trimer exhibit both homotropic and heterotropic effects (Gibbons et al., 1976). Moreover, the isomeric ATCase-like molecule in which the two active catalytic chains are in the same trimers exhibited similar catalytic properties (Gibbons et al., 1976). Hence the arrangement of the functional and inactive catalytic chains in the holoenzyme is unimportant, and the ligand-promoted allosteric transition involves the entire molecule.

In a recent study of the enzyme-catalysed arsenolysis of carbamoylaspartate it was shown that the holoenzyme is much less active than the isolated C subunit and that the substrates for the reverse reaction, unlike those for the physiological reaction, do not promote the T → R transition. Moreover, the binding of PALA to three of the six active sites in the enzyme caused a 40-fold increase in activity. These observations provided the basis for an active-site titration method which permitted the direct determination of the number of active sites in an ATCase molecule converted from the T- to the R-state upon the binding of only one PALA molecule (Krause & Schachman, 1985). The number was 4.7 for the enzyme in the presence of Mg·PP, a value very close to the theoretical maximum for a concerted transition with all of the active sites of the molecule changing from the T- to the R-state upon the binding of one PALA molecule.

Further evidence indicating that the allosteric transition is concerted, without the accumulation of significant amounts of intermediates, was obtained from an analysis of boundary spreading in sedimentation velocity experiments (W. E. Werner & H. K. Schachman, unpublished work). As indicated above, active-site ligands cause a progressive decrease in the sedimentation coefficient until the maximum value of about −3% is achieved. At intermediate PALA concentrations corresponding to about a 1.5% decrease, the sedimenting boundary was broader than that for either the T- or R-state enzyme. Quantitative analyses of the boundaries in terms of apparent diffusion coefficients showed that the solution contained a mixture of the two conformations of the enzyme. Moreover, the apparent diffusion coefficient for the half-converted wild-type enzyme was virtually identical to that for an equal mixture of fully liganded R-state wild-type enzyme and a mutant which did not bind PALA and was in the T-state. This experiment shows that the transition is concerted and that both T-state and R-state molecules exist together at sub-saturating PALA concentrations.

**Amino acid substitutions influence the homotropic effects**

The development of site-directed mutagenesis techniques provided an unusual opportunity to probe the effect of single amino acid replacements on both the allosteric properties and the enzyme activity of ATCase. Some substitutions (described below) yielded virtually inactive enzyme and others produced active enzyme with greatly altered allosteric properties (Robey et al., 1986). As seen in Fig. 4, replacing lysine-83 in the catalytic chains by glutamine leads to an enzyme with markedly reduced co-operativity, whereas the substitution of alanine for glutamine-133 in the same chains leads to enhanced co-operativity (Robey et al., 1986). As yet these mutant forms of ATCase have not been studied thoroughly in terms of the parameters describing the allosteric transition, but they will be useful as models for investigating the role of amino acid residues from adjacent polypeptide chains in a trimer. Functional residues are represented by the symbols Δ and Ω and it is assumed that site-directed mutagenesis can be used to

![Fig. 4. Effect of mutational alterations on the regulatory properties of ATCase](image-url)
Experiments have been conducted with two pairs of mutants. In one lysine 84 was replaced by glutamine, leading to a virtually inactive enzyme (Robey et al., 1986). The other mutants involved the substitution of alanine for histidine 134, resulting in an enzyme of much lower activity and a significantly increased $K_{m}$ (Robey et al., 1986) and the replacement of serine 52 by histidine to give a protein with virtually no activity (Y. R. Yang & H. K. Schachman, unpublished work). These particular mutants were selected not only because they were relatively inactive but also because in the trimer lysine 84 in one chain is at an interface (Krause et al., 1985) relatively close to the region of the adjoining chain containing both histidine 134 and serine 52. Thus lysine 84 could be representative of $\Delta$ and either histidine 134 or serine 52 would correspond to $\Box$. Incubating the pairs of mutants together under conditions favouring interchain exchange led to a very large increase in catalytic activity ($10^2$-$10^4$-fold). The purified hybrids from the glutamine 84 and alanine 134 mutants, on the one hand, and from the glutamine 84 and the histidine 52 mutants, on the other, had specific activities close to the expected 33% of the wild-type C trimers (Wente & Schachman, 1987). Similarly, the hybrids from the wild-type trimers and the double mutant containing glutamine 84 and alanine 134 had specific activities in excellent agreement with those predicted from the scheme in Fig. 5.

These complementation experiments with defective proteins produced by site-directed mutagenesis constitute a general method for detecting shared sites in oligomeric enzymes and demonstrate that ATCase forms its active sites by bringing into proper juxtaposition amino acid residues from adjacent chains. The results also show that monomers are intrinsically inactive and that active species can be formed only upon association of the chains to form oligomers.

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Fig. 5. Scheme illustrating the use of hybrids to identify shared active sites in an oligomeric enzyme

Large circles represent individual catalytic chains in a trimer; symbols $\Delta$ and $\Box$ correspond to active-site residues which are located in the different chains at the interface between them. Mutations in either residue are indicated by symbols $\blacklozenge$ and $\blacksquare$. Functional sites require adjoining $\Delta$ and $\Box$. In the upper diagram active hybrids are formed from two defective parental mutants. The lower diagram illustrates the loss in specific activity of a wild-type trimer when hybrids are formed between it and a double mutant.

form two inactive mutants either by converting $\Delta$ to $\blacklozenge$ or by changing $\Box$ to $\blacksquare$. If the two trimers are dissociated into monomers and then reconstitution occurs at random, a hybrid set composed of four species results as shown in Fig. 5. The two hybrids would each have one active site per trimer and purification of the hybrids would yield proteins with 33% of the specific activity of the wild-type trimers (Robey & Schachman, 1985). Since conformational corrections could occur leading to some restoration of activity, an additional experiment, illustrated in Fig. 5, could be performed by constructing hybrids from the wild-type trimer and the double mutant containing $\blacklozenge$ and $\blacksquare$. These hybrids shown in Fig. 5 would have one active site per trimer if the hybrid contained two wild-type chains and one chain with the two mutations. Similarly, the hybrid containing one wild-type chain and two double-mutant chains would have no active sites (Wente & Schachman, 1987). This negative complementation experiment, coupled with other controls, would provide strong evidence that the regeneration of active hybrids from the two defective proteins is attributable to the formation of functional shared sites.

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