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INTERACTION BETWEEN POLYPEPTIDE CHAINS WITHIN THE CATALYTIC SUBUNIT OF ASPARTATE TRANSCARBAMYLASE

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SUMMARY

The catalytic subunit of aspartate transcarbamylase, which normally exhibits Michaelis–Menten kinetics, gives rise to nonlinear double reciprocal plots of initial velocity data in the presence of the inhibitor ITP. The results of kinetic and binding studies on the enzyme in the presence of ITP are considered in conjunction with the fact that the catalytic subunit is composed of three polypeptide chains. The data are consistent with the proposition that the combination of carbamyl phosphate on one polypeptide chain can hinder the combination of ITP on other polypeptide chains, and *vice versa*. Thus the isolated catalytic subunit is capable of exhibiting a type of cooperative effect, although this effect is only observed in the presence of the inhibitor.

INTRODUCTION

Studies on the structure of native aspartate transcarbamylase indicate that it is composed of two catalytic subunits, each containing three similar or identical polypeptide chains, and three regulatory subunits, each composed of two polypeptide chains[1]. Interactions between the active sites on the catalytic subunits occur in the native enzyme, giving rise to nonlinear double reciprocal plots of initial velocity data. In contrast, it appeared that no such interactions occurred within the isolated catalytic subunit, for which only Michaelis–Menten kinetics were observed. However, in kinetic studies on the catalytic subunit it has now been shown that double reciprocal plots of initial reaction velocity with respect to the concentration of carbamyl phosphate become nonlinear in the presence of the inhibitor ITP. Kinetic and binding studies have been carried out to elucidate this effect, and it is concluded that interaction occurs between active sites on different polypeptide chains within the catalytic subunit.

MATERIALS AND METHODS

[8-^{14}C]ITP (24.4 Ci/mole) was purchased from Schwarz. All other reagents were obtained or prepared as described previously[2]. The catalytic subunit of aspartate transcarbamylase was prepared from the native enzyme according to Gerhart and Holoubek[3].

Measurement of enzyme activity

Enzyme activity was determined at 28 °C in the presence of 0.05 M *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid buffer (pH 8.0), at an enzyme concentration of 0.2 μg per ml. The carbamyl aspartate produced in 1-ml reaction mixtures was estimated as outlined previously[2]. To ensure that initial velocities were being measured, reactions were run for two time periods.

Binding experiments

The binding of ITP or carbamyl phosphate was measured at pH 8.0 in volumes of 0.2 ml containing 0.05 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid buffer, at an enzyme concentration of 0.5 mg/ml. The method of Paulus[4] was used, in conjunction with Visking membranes instead of UM 10 Diaflo membranes[5]. Data were analysed as previously described[5].

Counting of enzyme–ligand complex deposited on the membranes was performed in a Packard Tri-Carb liquid scintillation spectrometer, using Triton Scintillant. Blank values (obtained in the absence of enzyme) did not vary with the concentration of ITP, and corresponded to 0.6 μl of solution. Enzyme was completely retained by the membrane.

RESULTS AND DISCUSSION

Inhibition by ITP

ITP causes linear noncompetitive inhibition with respect to aspartate as the varied substrate (Fig. 1). These data were fitted to the equation

$$v = \frac{VA}{K_a (1 + I/K_{is}) + A (1 + I/K_{il})} \qquad (1)$$

using the appropriate computer program of Cleland[6]. The values obtained for K_{is} and K_{il} were 3.7 $\pm$ 0.6 mM and 5.8 $\pm$ 2.3 mM, respectively.

When carbamyl phosphate is the varied substrate, double reciprocal plots are linear in the absence of ITP, but become curvilinear in its presence (Fig. 2a). A further investigation of the effect of ITP was made over the higher part of the carbamyl phosphate concentration range, where curvature was not obvious in Fig. 2a. The results (Fig. 2b) show that there is a common point of intersection on the ordinate. Moreover, the slopes of the lines of Fig. 2b vary as a hyperbolic function of the ITP concentration.

The data of Fig. 2b were fitted to the equation

$$v = \frac{VA}{K_a \left\{ \frac{1 + I/K_{iN}}{1 + I/K_{iD}} \right\} + A} \qquad (2)$$

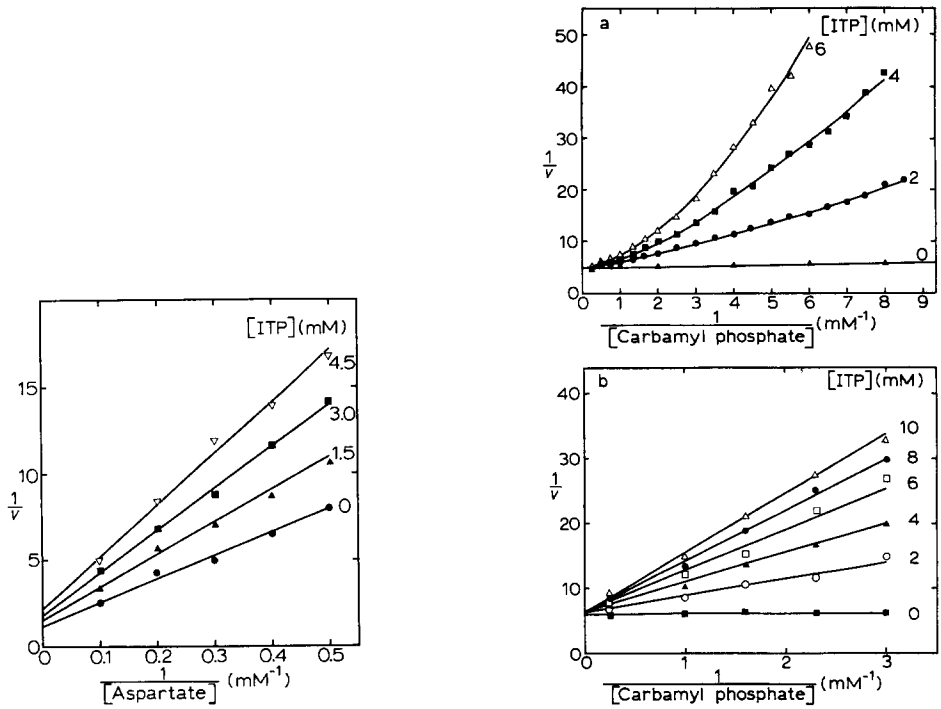

Fig. 1. Inhibition of the reaction by ITP, with aspartate as the varied substrate and the carbamyl phosphate concentration fixed at 0.5 mM. The concentrations of ITP were: ●, none; ▲, 1.5 mM; ■, 3.0 mM; ▽, 4.5 mM. The lines illustrate the fit of the data to Eqn 1. Velocity is expressed as μmoles of carbamyl aspartate formed per min per μg of enzyme.

Fig. 2. Inhibition of the reaction by ITP, with carbamyl phosphate as the varied substrate, and the aspartate concentration fixed at 5 mM. (a) The concentrations of ITP were: ▲, none; ●, 2 mM; ■, 4 mM; △, 6 mM. The lines illustrate the fit of the data at each fixed concentration of ITP to Eqn 3. (b) The concentrations of ITP were: ■, none; O, 2 mM; ▲, 4 mM; □, 6 mM; ●, 8 mM; △, 10 mM. The lines illustrate the fit of the data to Eqn 2. Velocity is expressed as μmoles of carbamyl aspartate formed per min per μg of enzyme.

by means of a computer program written by Cleland[6]. The values obtained for the inhibition constants were 0.16 ± 0.03 mM for K_{iN} and 23 ± 1 mM for K_{iD}. The data of Fig. 2a were fitted to the equation

$$v = \frac{V(A^2 + bA)}{A^2 + cA + d} \tag{3}$$

where b, c and d are constants, by means of a computer program written by Mr H. R. Kinns. The results of this analysis are given in Table I. The values for the constant b are doubtful, although the general fit indicates that the rate equation approximates to Eqn 3. It should be pointed out that this analysis is not the only way in which the data might be fitted to Eqn 3, since there are difficulties in obtaining a unique fit to an equation of such complexity.

The kinetic results are consistent with the combination of ITP either at the active site in direct competition with carbamyl phosphate, or at some other site, in

TABLE I

CONSTANTS DERIVED FROM INITIAL VELOCITY DATA OBTAINED IN THE PRESENCE OF ITP
The data obtained at each fixed concentration of ITP (Fig. 2a) were fitted to Eqn 3.

Concentration of ITP (mM)	*Value of constant (Eqn 3)*		
	b	*c*	*d*
2	0.059 ± 0.074	0.32 ± 0.04	0.042 ± 0.033
4	0.166 ± 0.052	0.33 ± 0.04	0.26 ± 0.05
6	0.010 ± 0.031	0.37 ± 0.09	0.21 ± 0.05

such a way that interactions occur between the carbamyl phosphate binding sites on the enzyme. ATP, UTP, CTP and GTP also cause inhibition of the enzyme, but apparently do not give rise to the cooperative effects observed with ITP. Studies on the inhibition of the reaction by ATP (ref. 2) indicate that this nucleotide functions simply as an analogue of carbamyl phosphate. It is therefore likely that ITP also combines at the carbamyl phosphate binding site.

Binding experiments

The binding of ITP appears linear both in the absence of carbamyl phosphate and when the concentrations of ITP and carbamyl phosphate are varied in constant ratio, and there is a decrease in the maximum number of moles of ITP bound per mole of enzyme when carbamyl phosphate is present (Fig. 3a). The constants derived from these data are recorded in Table II.

If ITP and carbamyl phosphate were to bind at separate sites on each polypeptide chain in the catalytic subunit, then a plot such as that in Fig. 3a (where the

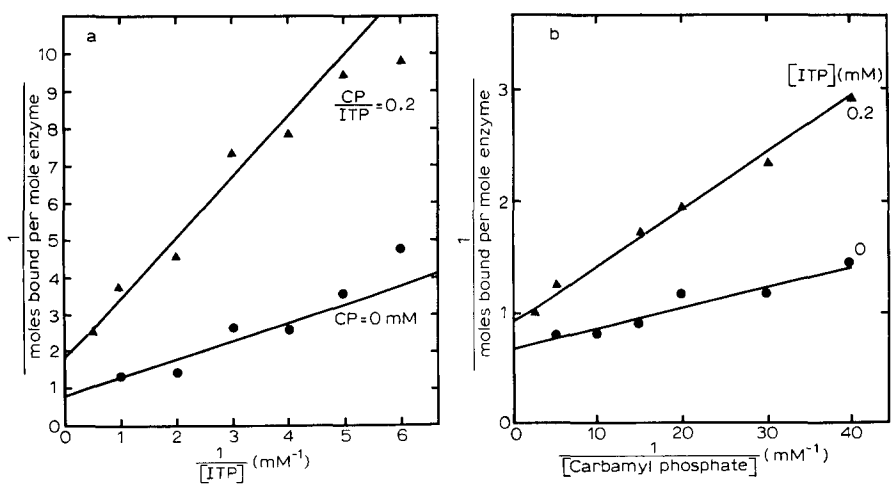

Fig. 3. Binding of ITP and carbamyl phosphate (CP) to the enzyme. (a) [^{14}C]ITP varied; ●, in the absence of carbamyl phosphate; ▲, with the carbamyl phosphate concentration varied at a fixed proportion (0.2) of the ITP concentration. (b) [^{14}C]Carbamyl phosphate varied; ●, in the absence of ITP; ▲, in the presence of 0.2 mM ITP. Further details are given in Materials and Methods.

TABLE II

BINDING CONSTANTS FOR ITP AND CARBAMYL PHOSPHATE

The data of Fig. 3 were analyzed as previously described[5]. The molecular weight of the catalytic subunit was taken as 100 000. Fully active enzyme was assumed to have a specific activity of 0.87 μmoles/min per μg at pH 8.0 (ref. 2).

Labelled ligand	*Unlabelled ligand*	*Dissociation constant (mM)*	*Maximum number of moles of ligand bound per mole of*	
			Enzyme protein	*Fully active enzyme*
ITP	—	0.48 ± 0.13	1.16 ± 0.13	2.1 ± 0.2
ITP	Carbamyl phosphate	0.84 ± 0.15	0.54 ± 0.05	0.96 ± 0.09
Carbamyl phosphate	—	0.027 ± 0.006	1.46 ± 0.09	2.58 ± 0.16
Carbamyl phosphate	ITP	0.055 ± 0.005	1.07 ± 0.04	1.89 ± 0.07

concentrations of ITP and carbamyl phosphate are varied in constant ratio) would be nonlinear, with the same ordinate intercept as when carbamyl phosphate is absent. The equation which applies is

$$1/\text{moles ITP bound per mole enzyme} = \frac{K_{ii}}{I} \cdot \frac{\left(1 + \frac{C}{K_{ic}}\right)}{\left(1 + \frac{C}{K_{ic}} \cdot \frac{K_{ii}}{K_{Ii}}\right)} + 1$$

where I represents ITP, C represents carbamyl phosphate, K_{ii} and K_{ic} are dissociation constants for the interactions of ITP and carbamyl phosphate, respectively, with free enzyme, and K_{Ii} is the dissociation constant for the interaction of ITP with the enzyme–carbamyl phosphate complex.

On the other hand, if ITP and carbamyl phosphate compete for the same site on each polypeptide chain then the upper line in Fig. 3a would be linear, with an ordinate intercept greater than that in the absence of carbamyl phosphate. The equation which applies in this case is

$$1/\text{moles ITP bound per mole enzyme} = K_{ii} \cdot \frac{1}{I} + \left(1 + \frac{C}{I} \cdot \frac{K_{ii}}{K_{ic}}\right)$$

where C/I is a constant.

The apparent linearity of the data in Fig. 3a, together with the decrease observed in the maximum number of moles of ITP bound when the concentration of carbamyl phosphate is simultaneously extrapolated to infinity, indicate that the latter hypothesis applies. That is, ITP and carbamyl phosphate are not bound simultaneously on each polypeptide chain. Rather, the results are consistent with direct competition by ITP for the carbamyl phosphate binding site.

The binding of carbamyl phosphate appears linear in both the absence and presence of a fixed concentration of ITP (Fig. 3b). It should be noted that, for practical reasons, the concentrations of carbamyl phosphate and ITP used in obtaining these binding data were lower than those used for the kinetic studies in which non-

linearity was observed (Fig. 2a). It might be expected that any nonlinearity of the plots would be more easily detected at the carbamyl phosphate concentrations of Fig. 3b than at those of Fig. 2a, but on the other hand the lower ITP concentration of Fig. 3b would make the detection of nonlinearity more difficult. The study of the effect of higher concentrations of ITP on the binding of carbamyl phosphate was not feasible. However, curvature is observed in kinetic data obtained at the carbamyl phosphate and ITP concentrations of Fig. 3b, using a fixed concentration of 0.2 mM aspartate and the assay for conversion of radioactive aspartate to carbamylaspartate which was employed for part of the kinetic studies on the reaction mechanism[2]. Thus if the reciprocal of the measured velocity at 0.2 mM carbamyl phosphate in the presence of 0.2 mM ITP is normalised to 1.25 (*i.e.* the reciprocal of the number of moles of carbamyl phosphate bound per mole of enzyme under these conditions in Fig. 3b), the corresponding values at 0.05 mM and 0.025 mM carbamyl phosphate would be 1.5 and 2.6, respectively, instead of the observed values of 1.95 and 2.9. It should have been possible experimentally to detect such curvature in the binding experiments if it had occurred. Therefore, the apparent linearity of the plot of carbamyl phosphate binding in the presence of ITP (Fig. 3b) indicates that ITP does not cause interaction between the sites at which carbamyl phosphate is bound. There appears to be a small difference in the vertical intercepts observed in Fig. 3b (Table II), whereas a common vertical intercept would have been expected on the basis of the kinetic data (Fig. 2). In view of the decreasing sensitivity associated with increasing ligand concentration in binding determinations, it is considered likely that the difference between the vertical intercepts in Fig. 3b is not significant. The precise positions of the lines depend on the weighting factors employed in analysing the data, and a small change in the slope of each line in Fig. 3b would be sufficient to give a common intercept. On the other hand, a fit by eye to the data of Fig. 3a would if anything tend to increase the vertical intercept discrepancy.

The directly determined values for the number of moles of carbamyl phosphate or ITP bound per mole of enzyme appear low when it is assumed that the enzyme has a molecular weight of 100 000 and is composed of three identical polypeptide chains[1], each of which presumably has an active site. In this connection, Collins and Stark[7] have recently reported the binding of three moles of a tight binding inhibitor per mole of catalytic subunit. However, we have found that some preparations of the enzyme are of lower specific activity than others, even though all appear homogeneous on polyacrylamide gel electrophoresis, and the specific activity of the preparation with which the present work was performed was 56% of the maximum ever observed in our hands, *viz.* 0.87 μmole/min per μg at pH 8.0 (ref. 2). If the present results are expressed as moles of ligand bound per mole of fully active enzyme they appear more consistent with the structure of the enzyme (Table II). However, there may be some other explanation for the apparently low number of moles of ligand bound per mole of enzyme. The early work on the binding of reactants by native aspartate transcarbamylase[8] indicated a lower number of moles bound per mole than might have been expected as a result of later work on the structure of the enzyme[1]. Moreover, there are examples of similar discrepancies with other enzymes for which different explanations have been advanced[9]. In this connection it should be noted that some enzymes are inactivated by carbamyl phosphate[10], although any effect on aspartate transcarbamylase appears to be insignificant under the conditions used in the present work.

In summary, the binding results are consistent with competition between ITP and carbamyl phosphate for the same sites on the enzyme. They indicate that there is no apparent cooperativity in the binding of carbamyl phosphate even when ITP is present.

A mechanism for the action of ITP

An explanation can be put forward for the effects induced by ITP, taking into account the kinetic and binding results in conjunction with the fact that the catalytic subunit is composed of a number of identical polypeptide chains. It is proposed that the combination of carbamyl phosphate on one polypeptide chain can cause a conformational change which hinders the combination of ITP at the active site on another polypeptide chain. Conversely, the combination of carbamyl phosphate with the enzyme would be hindered by the presence of ITP on another polypeptide chain. However, the presence of ITP at one active site need have no effect on the rate of the reaction which can still occur at a different active site.

For an enzyme molecule having two active sites and conforming to the above mechanism, reaction velocity in the presence of ITP would be the function of carbamyl phosphate concentration expressed in Eqn 3. However, for a molecule having three active sites the function would be more complex, involving terms in A^3, and it is not yet feasible to fit data to this equation. The catalytic subunit contains three polypeptide chains, and this may therefore be the reason why the data of Fig. 2a do not fit quite satisfactorily to Eqn 3.

Over the higher range of carbamyl phosphate concentration (Fig. 2b) the behaviour of the catalytic subunit approximates to that of an enzyme with a single active site, in the presence of a partial inhibitor which is not combining at that active site. (In this case, it is proposed that the separate site at which the inhibitor combines is actually a vacant carbamyl phosphate binding site on a different polypeptide chain.) On this simplified basis, where the concentration of the second substrate aspartate affects only the apparent values for V and K_a in Eqn 2 without altering the form of the equation, it would be expected that the kinetic constant K_{iN} of Eqn 2 would be identical with the dissociation constant determined from binding studies for the interaction of ITP with the free enzyme. The values determined for these two constants, 0.16 $\pm$ 0.03 mM and 0.48 $\pm$ 0.13 mM, respectively, are of the same order of magnitude. The constant K_{iD} of Eqn 2 would correspond under these conditions to the dissociation constant for the reaction of ITP with the enzyme when carbamyl phosphate is present on another active site. The value for K_{iD}, 23 $\pm$ 1 mM, is much greater than that for K_{iN}. Similarly, the combination of carbamyl phosphate would be markedly hindered when ITP is present on another polypeptide chain. The linearity of the double reciprocal plots in Fig. 1, obtained at a relatively high fixed concentration of carbamyl phosphate, is also consistent with the proposed mechanism for ITP inhibition. However, no physical significance can be attributed to the apparent inhibition constants determined from the data of Fig. 1.

Thus interactions can occur between active sites on different polypeptide chains within the catalytic subunit. It appears as though the effects are manifested only in the presence of ITP. Nevertheless, it seems unlikely that such interactions would occur only with the one reactant, but rather that they are too small to be detected with other reactants.

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