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A UNIFYING CONCEPT FOR THE ACTIVE SITE REGION IN ASPARTATE TRANSCARBAMYLASE

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Summary

Recent investigations on the aspartate transcarbamylases (carbamoylphosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) of *Escherichia coli* and *Streptococcus faecalis* indicate that there is a site on each enzyme, apart from the active site, at which anions can bind. It is suggested in this paper that the location of such an anion binding site on the *E. coli* enzyme may be directly adjacent to the part of the active site at which carbamyl phosphate binds. This hypothesis is based on data demonstrating a lack of correlation between spectral changes and kinetic effects, and on a new interpretation of results obtained with *N*-(phosphonacetyl)-L-aspartate, which has previously been considered to act as a transition state analogue. Such a hypothesis could explain other puzzling observations made on the catalytic subunit of this enzyme, including the dependence of substrate inhibition by aspartate on the nature of the second substrate, and the ease of formation of a dead-end enzyme-aspartate-carbamyl-aspartate complex.

Introduction

There is discussion in the literature as to whether the aspartate transcarbamylase reaction catalysed by the enzyme from *Escherichia coli* (carbamoylphosphate: L-carbamoyltransferase, EC 2.1.3.2) involves a random addition and release of reactants, or an ordered sequence in which aspartate binds after carbamyl phosphate and carbamylaspartate is released before phosphate. The simplest mechanism consistent with the steady-state kinetic and binding data is the random mechanism. However, results obtained with a compound proposed as a transition state analogue for the reaction indicate that the active site region is more complex than expected on the basis of either mechanism. The hypothesis being put forward has the potential to resolve apparently conflicting evidence

concerning the reaction mechanism and the active site region in the catalytic subunit, and is one which could be tested in the course of X-ray crystallographic studies that are currently in progress [1].

Discussion of Background and Results

The first kinetic investigation of the reaction mechanism [2] gave a product inhibition pattern that was qualitatively consistent either with the ordered mechanism or with a random mechanism involving the formation of dead-end complexes, but the data were interpreted only in terms of the ordered mechanism. Subsequently, Collins and Stark [3] synthesized *N*-(phosphonacetyl)-L-aspartate (Fig. 1) which was considered to be a transition state analogue, and found that it caused competitive inhibition with respect to carbamyl phosphate but non-competitive inhibition with respect to aspartate. Since a transition state analogue would be expected to cause competitive inhibition with respect to both substrates if the mechanism were random, it was argued [3] that the *N*-(phosphonacetyl)-L-aspartate results confirmed the ordered mechanism. However, it will be shown that there is reason to question whether *N*-(phosphonacetyl)-L-aspartate really functions as a transition state analogue.

Later kinetic studies involving product and substrate analogue inhibition, binding studies and isotope transfer studies [4,5] indicated that the simple ordered mechanism was ruled out on quantitative grounds. The results were consistent with a random mechanism involving three dead-end complexes and occurring under what approximated to rapid equilibrium conditions, although there were indications that these conditions might not apply strictly. The evidence against an ordered mechanism is summarized by Heyde et al. [4]. It includes the facts that succinate, an analogue of aspartate, causes non-competitive rather than uncompetitive inhibition with respect to carbamyl phosphate, and that aspartate is bound to the enzyme in the absence of carbamyl phosphate, with a dissociation constant consistent with the kinetically determined dissociation constant. Moreover, carbamylaspartate was bound well to the enzyme in the absence of phosphate, which would not be expected if it were the first product released in an ordered sequence. In addition it was observed that both aspartate and *N*-(phosphonacetyl)-L-aspartate could bind at the same time to the enzyme, each with a spectral dissociation constant consistent with

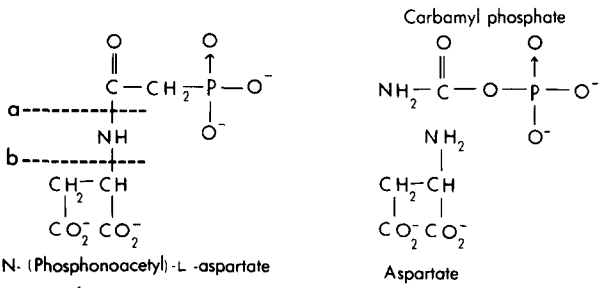

Fig. 1. Structures of the substrates for the aspartate transcarbamylase reaction and of the proposed transition state analogue, *N*-(phosphonacetyl)-L-aspartate.

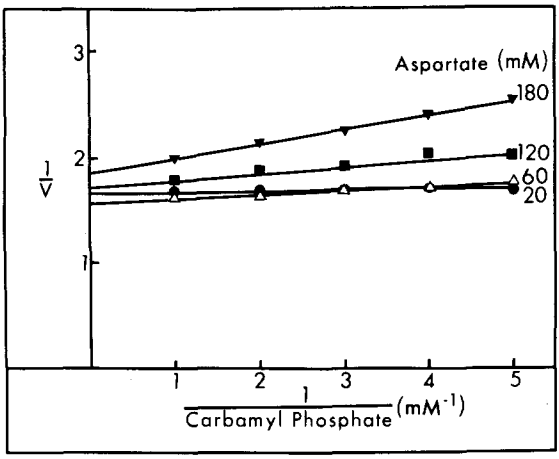

Fig. 2. Substrate inhibition by aspartate at pH 8.0. Assays were performed at 28° C in 0.05 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) buffer, pH 8.0, by colorimetric determination of the carbamylaspartate formed. The data for each line were fitted separately using the HYPER computer program. Reagents and methods were as previously described [4]. v is expressed as μmol of carbamylaspartate formed per min per μg of catalytic subunit.

the corresponding dissociation constant determined kinetically [4,6].

The only other study bearing directly on the reaction sequence is that by Wedler and Gasser [7]. They recently claimed to demonstrate ordered substrate binding for the catalytic subunit by means of isotope exchange studies at equilibrium, in spite of the high equilibrium constant for the reaction. However, even if rapid equilibrium conditions do not apply in a random reaction mechanism so that the rates for the two exchanges are not equal, random addition of the substrates aspartate and carbamyl phosphate is not precluded unless the rate of the slower exchange falls to zero at higher levels of reactants, and this has not been demonstrated. Moreover, the experimental detail given for their work raises problems. Thus it appears that the chemical concentration of carbamyl [^{32}P]phosphate added (Fig. 2 of ref. 7) was very high compared with the equilibrium concentration of carbamyl phosphate (6 mM cf. < 10 μM), and this addition of label would shift the equilibrium. Hence the exchange measurements would not have been made at equilibrium. Moreover, even if the concentration of carbamyl [^{32}P]phosphate is assumed to be negligible, the concentrations of reactants indicated in the legend to Fig. 2 [7] do not satisfy the equilibrium relationship. The concentration of carbamyl phosphate would be two to three orders of magnitude lower by the time equilibrium was established, and the different conditions used to measure the two exchanges markedly favour the rate of the aspartate-carbamylaspartate exchange over that for the carbamyl phosphate-phosphate exchange. Similar criticism applies to the results reported for the native enzyme.

Thus the results obtained with *N*-(phosphonacetyl)-L-aspartate provide the only reliable experimental evidence which is, on the assumption that *N*-(phosphonacetyl)-L-aspartate is a transition state analogue, inconsistent with a random reaction mechanism. However, for both ordered and random mecha-

nisms the observed independent binding of both *N*-(phosphonacetyl)-L-aspartate and aspartate to the enzyme at the same time [4,6] is unexpected. This observation is not consistent with the reaction of both compounds within the normal active site. The recent studies of Jacobson and Stark [6] on the binding of dicarboxylic acids and other anions to the enzyme are relevant to this point. But first it should be noted that our data are at variance with the statement by these authors that α-methyl-DL-aspartate does not inhibit competitively with respect to aspartate. (This conclusion is based apparently on the data of Davies et al. [18]). In the present work α-methyl-DL-aspartate (from Sigma) was observed to be a competitive inhibitor in relation to aspartate, in 0.05 M tris-(hydroxymethyl)methylaminoethane sulphonic acid buffer, pH 8.0, with the carbamyl phosphate concentration fixed at 1 mM, giving an inhibition constant of 184 ± 11 mM from analysis of the data using the COMP computer program [4]. Thus all the dicarboxylic acids tested as analogues of aspartate cause competitive inhibition with respect to aspartate. Jacobson and Stark [6] determined dissociation constants for the reaction of a number of dicarboxylic acids and other anions with the enzyme, in the presence and absence of *N*-(phosphonacetyl)-L-aspartate or carbamyl phosphate, by means of binding (difference spectral) and kinetic techniques. A summary of relevant constants for some dicarboxylic acids, including the substrate aspartate, is given in Table I. All the values represent true rather than apparent constants, with the basis for any calculations involved being given in the appropriate references.

If the dissociation constant for the reaction of a compound at the active site is measured by both techniques the two values in Table I ought to agree. Such agreement is observed for the substrate aspartate, but is not observed for α-methyl-DL-aspartate, which inhibits the enzyme weakly with a K_i of approx. 200 mM under conditions where its spectral dissociation constant is approx. 20 mM. A second example where a discrepancy exists between values obtained by the two techniques lies in the succinate data in Table I. Thus for the interaction of succinate with the free enzyme the kinetic dissociation constant given by Jacobson and Stark [6] is approx. 3.5 mM while the spectral dissociation constant is 20—25 mM. While uniform conditions have not been used in all the studies from which Table I is compiled, each pair of values compared above was obtained under directly comparable conditions with respect to pH and type of buffer. It is also important to note that the values of the kinetic constants for both α-methyl-DL-aspartate and succinate in the above discussion are unaffected by whether the calculation is based on a random or an ordered mechanism. In the latter case it is assumed that succinate reacts with free enzyme as well as the enzyme-carbamyl phosphate complex.

The discrepancies between the spectral and kinetic dissociation constants for succinate and α-methyl-DL-aspartate indicate that there is an anion binding site different from the part of the active site at which aspartate and its analogues normally combine. Jacobson and Stark [6] have proposed that dicarboxylic acids cause difference spectra by combining at the carbamyl phosphate binding site, but this explanation cannot apply at least for aspartate, α-methyl-DL-aspartate and succinate. Thus it follows from their proposal [6] that anions causing difference spectra, including aspartate, should cause inhibition as a result of combination at the carbamyl phosphate site. Aspartate does cause substrate

TABLE I

DISSOCIATION CONSTANTS FOR THE INTERACTION OF DICARBOXYLIC ACIDS WITH THE CATALYTIC SUBUNIT OF ASPARTATE TRANSCARBAMYLASE

The number in parentheses after each value is the reference from which the constant was obtained. The thermodynamic constants were determined using difference spectra, except for constants from ref. 4, which were determined by an ultrafiltration method. Constants from ref. 4 and 5 and the present work were determined at pH 8.0 and 28°C in 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid buffer. Constants given in ref. 6 were determined at pH 7.0 and 28°C in 10 mM imidazole/5 mM acetate buffer with 2 mM 2-mercaptoethanol and 0.2 mM EDTA except where footnotes indicate otherwise.

Reactant	Enzyme (E) form reacted with	Dissociation constant (mM)	
		Kinetic	Thermodynamic
Aspartate	E	22 ± 6 (5)	15 (4), 15 (6)
	E-carbamyl phosphate	8.1 ± 0.5 (4)	
	E-N-(phosphonacetyl)-L-aspartate		20 (6)
	E-carbamylaspartate	2.7 ± 0.8 (4)	
α-Methyl-DL-aspartate	E	42 ± 5 [a] (6)	20 (6)
	E-carbamyl phosphate	>200 [b] (6), 184 ± 11 [c]	20 (6)
Succinate	E	3.5 ± 1 [a] (6), 0.3 ± 0.1 (4), 6 ± 2 (5)	20 [d] (6), 25 (6)
	E-carbamyl phosphate	3.5 [e] (6), 1.0 ± 0.2 (4)	0.74 [f] (9)
	E-N-(phosphonacetyl)-L-aspartate		300 ± 50 (6)
Carbamylaspartate	E		1.5 ± 0.2 (4)
	E-aspartate	<0.21 (4)	
	E-carbamyl phosphate	28 ± 2 (4)	
N-(phosphonacetyl)-L-aspartate	E	1.6×10^{-3} [g] (6)	
	E-aspartate		1.4×10^{-3} [h] (6)

[a] pH 8.0, 90 mM Tris/acetate, 1 mM 2-mercaptoethanol.
[b] pH 7.0, 100 mM imidazole acetate.
[c] pH 8.0, 50 mM tris(hydroxymethyl)methylaminoethanesulphonic acid (present work).
[d] pH 8.0, 10 mM Tris/5 mM acetate, 2 mM 2-mercaptoethanol and 0.2 mM EDTA.
[e] pH 7.9, 200 mM glycylglycine.
[f] pH 7.0, 20 mM glycylglycine, 2 mM 2-mercaptoethanol, 0.02 mM EDTA.
[g] pH 7.0, 100 mM imidazole acetate with 1 mM 2-mercaptoethanol and 400 mM lysine acetate. This value was determined under conditions comparable with those used to determine the thermodynamic value given for *N*-(phosphonacetyl)-L-aspartate. At lower ionic strength the kinetic value is $2.7 \cdot 10^{-5}$ mM [3]. In inhibition experiments *N*-(phosphonacetyl)-L-aspartate did not function kinetically as a tight-binding inhibitor because the enzyme concentration was 2—3 orders of magnitude lower than the *N*-(phosphonacetyl)-L-aspartate concentration.
[h] pH 7.0, 100 mM imidazole acetate with 200 mM aspartate.

inhibition of pH 8.0, when carbamyl phosphate is the other substrate, at concentrations which are high relative to its spectral dissociation constant (Fig. 2), but there are three features of the inhibition which are inconsistent with the hypothesis that the inhibition and spectral change are caused by combination at the carbamyl phosphate site. Firstly, inhibition is not competitive with respect

to carbamyl phosphate (Fig. 2). Secondly, inhibition does not occur at pH 7.0 [3] even though aspartate causes a spectral change at pH 7.0, and thirdly, inhibition does not occur even at pH 8.0 if carbamyl phosphate is replaced as a substrate by acetyl phosphate [5]. Moreover, if succinate and α-methyl-DL-aspartate combine at the carbamyl phosphate site under conditions where aspartate does not, they ought to cause non-competitive inhibition with respect to aspartate. In fact, succinate causes linear competitive inhibition [4,5] and α-methyl-DL-aspartate also causes competitive inhibition, the linearity of which could not be determined because the inhibition was too weak.

General discussion

The results obtained with *N*-(phosphonacetyl)-L-aspartate, considered as a transition state analogue, have played a prominent part in discussions on the mechanism of the aspartate transcarbamylase reaction [3,4,6,10]. The mechanism is commonly assumed on this basis to be ordered, and experimental design and interpretation based on this assumption may be causing confusion and error in this field if the interpretation of the *N*-(phosphonacetyl)-L-aspartate results is incorrect. Since the proposed function of *N*-(phosphonacetyl)-L-aspartate as a transition state analogue is at variance with the simplest reaction mechanism that fits the other steady-state kinetic and binding data, it must be questioned.

N-(phosphonacetyl)-L-aspartate has so far been considered [3,10] to be composed of moieties resembling carbamyl phosphate lacking the amino group and aspartate (Fig. 1a), but it can also be considered to resemble the complete carbamyl phosphate molecule together with succinate (Fig. 1b); and, unlike aspartate, succinate does not have a simple relationship between its kinetic and thermodynamic binding constants (Table I). The tight binding of *N*-(phosphonacetyl)-L-aspartate relative to carbamyl phosphate or succinate certainly indicates that both moieties are bound to the enzyme [6]. However, it may well be that the combination of the succinate moiety is not at the aspartate binding part of the active site as argued previously [3,6,10], but rather at a separate anion binding site adjacent to the carbamyl phosphate site. In this case *N*-(phosphonacetyl)-L-aspartate would not be acting as a true transition state analogue, but its binding would be increased over that of either substrate.

The fact that *N*-(phosphonacetyl)-L-aspartate and aspartate can combine with the enzyme at the same time, each with a spectral dissociation constant consistent with the corresponding kinetic constant, has two possible explanations. Either aspartate combines to give a spectral dissociation constant elsewhere than at the active site, and agreement of this constant with the kinetic constant is fortuitous, or aspartate combines to give a spectral change at the aspartate binding part of the active site and *N*-(phosphonacetyl)-L-aspartate is not a true transition state analogue, but combines as suggested above, at both the carbamyl phosphate binding site and a separate but adjacent anion binding site. Jacobson and Stark [6,10] favour the former interpretation, which necessarily disregards the kinetic and binding evidence in favour of a random reaction mechanism and against an ordered mechanism [4,5], whereas the latter interpretation removes all inconsistencies. The correct interpretation

could be discerned by means of X-ray crystallography of the catalytic subunit in the presence of both *N*-(phosphonacetyl)-L-aspartate and aspartate. Investigations of the crystallographic structure of this enzyme are already well advanced [1].

There is evidence that the site at which succinate is bound to free enzyme, causing a spectral change, overlaps the *N*-(phosphonacetyl)-L-aspartate binding site. Thus the spectral dissociation constant for succinate is markedly increased in the presence of *N*-(phosphonacetyl)-L-aspartate (Table I). In contrast, that for aspartate is virtually unaffected (Table I). This difference between the results for succinate and aspartate is consistent with the relationships between the dissociation constants observed for these two compounds in the absence of *N*-(phosphonacetyl)-L-aspartate (Table I). Thus, the kinetic and spectral dissociation constants for the interaction of the compounds with free enzyme are in agreement for aspartate but not for succinate, and the most direct conclusion from this is that the spectral change observed with aspartate reflects combination at the active site while that caused by succinate does not. All the data are consistent with the combination of succinate with the free enzyme at an anion binding site different from the aspartate binding part of the active site, but directly adjacent to the carbamyl phosphate binding site and forming part of the binding site for *N*-(phosphonacetyl)-L-aspartate. It may also combine at the aspartate binding part of the active site, but there is no evidence that a spectral change results from this combination.

Two other puzzling observations might be explained on the basis of an anion binding site adjacent to the carbamyl phosphate binding site, viz. the dependence of substrate inhibition by aspartate on the nature of the second substrate, and the ease of formation of a dead-end enzyme-aspartate-carbamyl-aspartate complex in a random reaction mechanism. In relation to the first point, aspartate may combine at the anion binding site, at pH 8.0 in the presence of carbamyl phosphate, to cause inhibition; it is conceivable that an unfavourable change in that site could be induced by the adjacent binding of acetyl phosphate or by lower pH, so that inhibition cannot occur. In this connection it should be noted that the presence of succinate alters the spectrum produced by carbamyl phosphate, but not that produced by acetyl phosphate [9]. With regard to the second point, it is not at all unusual for dead-end complexes to form in a random reaction mechanism [11] and for the catalytic subunit of aspartate transcarbamylase the simplest mechanism consistent with the steady-state kinetic and binding data involves three such complexes, viz. enzyme-aspartate-phosphate, enzyme-carbamyl phosphate-carbamylaspartate and enzyme-aspartate-carbamylaspartate [4]. What is striking is that the interaction of carbamylaspartate with the enzyme-aspartate complex (or of aspartate with the enzyme-carbamylaspartate complex) appears to be stronger by approximately an order of magnitude than its interaction with free enzyme (ref. 4 and Table I). Such a result is consistent with the combination of carbamylaspartate through its carbamyl moiety at the active site (carbamyl phosphate binding site) and through its aspartate moiety at an anion binding site adjacent to the carbamyl phosphate binding site but different from the aspartate binding part of the active site, i.e. in a manner similar to that proposed for *N*-(phosphonacetyl)-L-aspartate. If the binding of aspartate at the active site

enhances the combination of carbamylaspartate and vice versa, the observed kinetic result would be produced. It should be noted that the kinetic data indicate that aspartate does enhance the binding of carbamyl phosphate and vice versa [4].

The observations concerning an anion binding activator site distinct from the active site in *Streptococcus faecalis* aspartate transcarbamylase [12–14] give indirect support for the plausibility of the hypotheses raised above. There is kinetic evidence that the enzymes from both sources catalyze the reaction by means of a random reaction mechanism involving a dead-end enzyme-carbamyl phosphate-carbamylaspartate complex [4,13]. Chang and Jones [13] have suggested as a possibility for the ready formation of this complex that one moiety of carbamylaspartate could combine with the aspartate binding part of the active site while the other moiety interacts with the activator site.

Thus there is consistency between the results for aspartate transcarbamylase from both *E. coli* and *S. faecalis* and the prediction for a model with an anion binding site adjacent to the carbamylphosphate binding site. Perhaps the function of such an anion-binding site could have been lost in the *E. coli* enzyme during the evolution of the role of the regulatory subunits.

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