Biochimica et Biophysica Acta, 662 (1981) 8–14
Elsevier/North-Holland Biomedical Press

BBA 69379

COMPARISON OF THE ESSENTIAL ARGININE RESIDUE IN *ESCHERICHIA COLI* ORNITHINE AND ASPARTATE TRANSCARBAMYLASES

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(Received February 4th, 1981)
(Revised manuscript received June 10th, 1981)

Key words: Arginine residue; Ornithine transcarbamylase; Aspartate transcarbamylase; (E. coli)

The reaction of phenylglyoxal with *Escherichia coli* ornithine transcarbamylase (carbamoylphosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3) leads to complete loss of enzymatic activity. The behavior of this reagent towards ornithine transcarbamylase is remarkably similar to that observed with *E. coli* aspartate transcarbamylase (carbamoylphosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) and its catalytic subunit (Kantrowitz, E.R. and Lipscomb, W.N. (1976) J. Biol. Chem. 251, 2688–2695). The rate of phenylglyoxal inactivation increases in the order ornithine transcarbamylase, catalytic subunit of aspartate transcarbamylase and aspartate transcarbamylase. For ornithine transcarbamylase, the substrate carbamyl phosphate alone or in combination with the substrate analog norvaline protect the enzyme from phenylglyoxal inactivation. Under similar conditions, carbamyl phosphate or carbamyl phosphate plus succinate will protect the catalytic subunit of aspartate transcarbamylase in an almost identical manner. Using [^{14}C]phenylglyoxal, the number of arginine residues involved in loss of activity was determined to be approx. three per ornithine transcarbamylase molecule or one arginine per active site. The data suggest that the arginine necessary for activity is involved in the binding of carbamyl phosphate to the enzyme. The similarity in phenylglyoxal reactivities combined with genetic and structural data suggest very strongly that there is an evolutionary relationship between ornithine transcarbamylase and the catalytic subunit of aspartate transcarbamylase.

Introduction

Although the enzymatic transfer of a carbamoyl group is not a very common reaction, it does occur in a number of critical metabolic pathways. For example, the enzyme aspartate transcarbamylase catalyzes the carbamoylation of aspartate in the pyrimidine pathway, and ornithine transcarbamylase catalyzes the carbamoylation of ornithine in the arginine biosynthesis pathway. A cursory comparison of these two enzymes from *Escherichia coli* would suggest that the 310 000-dalton aspartate transcarbamylase dodecamer has little in common with the 105 000-dalton ornithine transcarbamylase trimer. However, when the intact aspartate transcarbamylase molecule is treated by a variety of means, including heat or reaction with mercurials [1], it dissociates into two 100 000-dalton subunits which retain catalytic activity, and three 33 000-dalton subunits which have no catalytic activity but bind the enzyme's regulatory nucleotides. The larger or catalytic subunits are trimers while the smaller or regulatory subunits are dimers [2,3]. The catalytic subunit of aspartate transcarbamylase has approximately the same molecular weight and quarternary structure as does ornithine transcarbamylase [4]. Furthermore, both of these species exhibit Michaelis-Menten kinetics, in contrast to the sigmoidal kinetics manifested by the native aspartate transcarbamylase [5].

Of these two enzymes, aspartate transcarbamylase

has been more extensively studied [6,7]. By chemical modification techniques, amino acid residues such as lysine [8], histidine [8], tyrosine [9] and arginine [10] have been found to be necessary for catalytic activity and are located at the active site of the enzyme. In particular, the reagent phenylglyoxal has been shown to very rapidly cause loss of enzymic activity and furthermore, this loss of activity can be directly correlated with the loss of a single arginine residue per active site [10]. Here, we use this reagent to determine if *E. coli* ornithine transcarbamylase contains an essential arginine residue, and if such a residue exists, to compare it's reactivity towards phenylglyoxal with the essential arginine residue of *E. coli* aspartate transcarbamylase. These data should help to establish how closely these enzymes are related to each other and how similar their active sites are.

Experimental

Materials. Sephadex G-25, G-150, A-50 and AH-Sepharose 4B were obtained from Pharmacia. L-Ornithine, L-citrulline, L-aspartate, carbamyl-L-aspartate, L-norvaline, succinate, phenylglyoxal, aspartate and carbamyl phosphate were purchased from Sigma Chemical Company. The carbamyl phosphate was purified by precipitation from 50% ethanol and stored desiccated at $-20°$ [11]. [^{14}C]Phenylglyoxal was synthesized by the method of Riley and Gray [12] from [^{14}C]acetophenone, which was obtained from New England Nuclear. The purified [^{14}C]phenylglyoxal had a specific activity of $2.0 \cdot 10^{11}$ cpm/mol.

Bacterial strains. The bacterial strain used in this study was specially constructed so that it would not only overproduce ornithine transcarbamylase but would also produce an enzyme derived only from the *argI* structural gene. Our starting strain 3000X111 [13] *thi, relA,* Δ(*proB-lac*), λ^- was obtained from the *E. coli* Genetic Stock Center, Yale University (No. 5263). Since this strain carries a deletion in the *proB-lac* region which includes *argF,* it possesses only one structural gene for ornithine transcarbamylase (*argI*). Isolation of an *argR* version of this strain was accomplished by selecting mutants resistant to 100 μg/ml L-canavanine sulfate in AF media [14] on plates. Overproduction of *argI* ornithine transcarbamylase was checked by direct enzyme activity measurements of sonicated cell extracts derived from the *argR* and parent strains. There was approx. a 40-fold increase in ornithine transcarbamylase in the *argR* strain.

Growth and purification. Ornithine transcarbamylase (*argI*) was isolated from 8 l *E. coli* K12 using modifications of the literature procedure for ornithine transcarbamylase from *E. coli* W [15]. In particular, Sephadex G-150 superfine was substituted for the G-200 superfine in the molecular weight step and the ornithine transcarbamylase was eluted form the AH-Sepharose column by an 0.25 M KCl wash. After purification the enzyme was stored in 0.04 M potassium phosphate buffer/2 mM 2-mercaptoethanol/0.2 mM EDTA, pH 7.0.

Aspartate transcarbamylase was isolated from *E. coli* K12 by the procedure of Gerhart and Holoubek [16]. The enzyme was stored under sterile conditions in 0.04 M phosphate buffer/2 mM 2-mercaptoethanol/0.2 mM EDTA, pH 7.0 in sealed vials under nitrogen at 5°C. The catalytic subunit was isolated by chromatography on DEAE Sephadex A-50 after dissociation of the native enzyme with *p*-hydroxymercuribenzoate by procedures previously reported [10]. The catalytic subunit of aspartate transcarbamylase was stored as an $(NH_4)_2SO_4$ precipitate. The purity of the enzyme was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [17]. Before use, the enzymes were exhaustively dialyzed against 0.125 M potassium bicarbonate, pH 8.3.

Enzyme concentrations were determined by absorbance measurements at 280 nm assuming an extinction coefficient of 0.59 cm^2/mg for native aspartate transcarbamylase [16], and 0.72 cm^2/mg for the isolated catalytic subunit [16]. For phenylglyoxal modified enzyme and all work with ornithine transcarbamylase protein was determined by the method of Lowry et al. [18], or the Bio-Rad version of Bradford's dye-binding assay [19] was used.

Measurement of enzyme activity. The transcarbamylase activity was measured by either a colorimetric or a pH-stat assay. The colorimetric assay was based on the 'Method II' procedure of Prescott and Jones [20] with modifications similar to those previously described [21]. pH-Stat assays were carried out with a Radiometer TTT80 Titrator and a ABU80 autoburette.

All assays were performed employing 4.8 mM car-

bamyl phosphate and either 10 mM ornithine or 30 mM aspartate. For the colorimetric assay, the reaction was carried out in 0.05 M Tris-acetate buffer, pH 8.3.

Activities in the figures are reported as percent activity relative to the activity at time zero of the phenylglyoxal reaction.

Modification with phenylglyoxal. Ornithine and aspartate transcarbamylase were reacted at 25°C with varying concentrations of phenylglyoxal in 0.125 M potassium bicarbonate, pH 8.3. At specific time intervals, 25-μl aliquots of the solution were removed and added to between 0.5 and 1.0 ml ice-cold 1.0 M Tris-acetate, pH 8.3, to quench the modification reaction. The colorimetric assay was performed to determine the extent of activity loss due to the phenylglyoxal. Likewise, the reaction was performed in the presence of carbamyl phosphate, and carbamyl phosphate plus substrate analogs.

[^{14}C]Phenylglyoxal was utilized to determine the number of arginine residues modified per polypeptide chain. At various times, 200 μl aliquots of the reaction mixture were passed over a 0.9 X 12.5 cm column of Sephadex G-25 using 0.05 M ammonium bicarbonate pH 8.5 as eluent to quench the modification reaction. The enzyme-containing fractions were pooled before division into aliquots for the immediate determination of enzymatic activity and subsequent analysis of protein concentration and radioactivity. Radioactivities were determined by mixing 25–50 μl of sample with 10 ml scintiverse (Fisher Scientific Company) and counting in a Nuclear Chicago Unilux II liquid scintillation counter. The number of arginine residues modified was calculated using a stoichiometry of two phenylglyoxal molecules per arginine residue modified [28].

Data analysis. Fitting the experimental data to kinetic equations was accomplished by a fitting and plotting program written in Basic and implemented on a Hewlett-Packard 85 computer.

Results

Inactivation of ornithine transcarbamylase by phenylglyoxal. All enzymatic activity of ornithine transcarbamylase is destroyed upon reaction with phenylglyoxal at pH 8.3 in 0.125 M potassium bicarbonate buffer. The inactivation reaction is dependent on the phenylglyoxal concentration, although under our experimental conditions the reactions are pseudo-first-order. Fig. 1 shows the dependence of the inactivation rate upon the phenylglyoxal concentration. A plot of pseudo-first-order rate constant versus phenylglyoxal concentration (see inset) was used to determine a second-order rate constant of 4.6 $M^{-1} \cdot min^{-1}$.

Comparison of phenylglyoxal inactivation of ornithine transcarbamylase, aspartate transcarbamylase, and the catalytic subunit of aspartate transcarbamylase. Fig. 2 shows the rate of phenylglyoxal inactivation of ornithine transcarbamylase, aspartate transcarbamylase and the catalytic subunit of aspartate transcarbamylase under identical conditions. At pH 8.3 in potassium bicarbonate buffer, the phenylglyoxal inactivation of ornithine transcarbamylase is 6.1-times slower than for aspartate transcarbamylase and 3.6-times slower than for the catalytic subunit of aspartate transcarbamylase.

Protection against inactivation by substrates and substrate analogs. The rate at which phenylglyoxal inactivates ornithine transcarbamylase is slowed when the modification reaction is carried out in the presence of substrates or substrate analogs. As seen in Fig. 3A, when the phenylglyoxal reaction is allowed to proceed in the presence of saturating carbamyl phosphate the inactivation of the enzyme is slowed by a factor of 3.8. In the presence of carbamyl phosphate plus norvaline, a nonreactive analog of ornithine, additional protection from inactivation was afforded the enzyme. Under these conditions, the reaction was slowed by a factor of 8.2.

As seen in Fig. 3B, the presence of substrate and substrate analogs similarly affected the rate of phenylglyoxal inhibition of the catalytic subunit of aspartate transcarbamylase. The substrate carbamyl phosphate slowed the inactivation rate by a factor of 3.8 while the combination of carbamyl phosphate and succinate slowed the rate by a factor of 9.5. As has been previously reported, aspartate transcarbamylase and its catalytic subunit are protected in almost identical fashion by substrates and substrate analogs [10].

Incorporation of [^{14}C]phenylglyoxal. In order to determine the number of arginine residues modified, radioactive phenylglyoxal was used. The reaction between ornithine transcarbamylase and [^{14}C]phenyl-

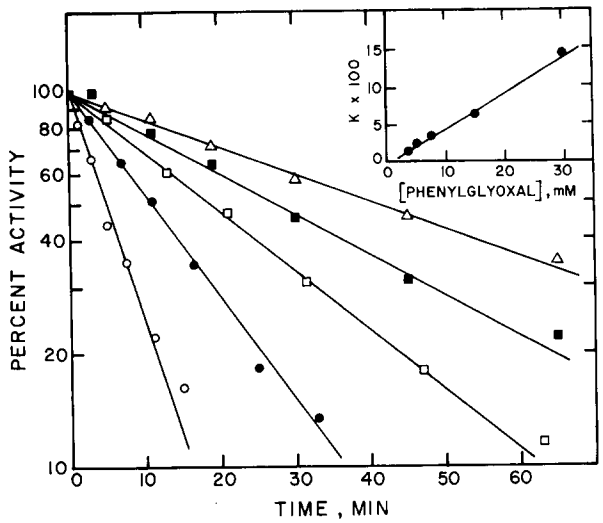

Fig. 1. Inactivation of ornithine transcarbamylase by phenylglyoxal. Effect of varying the concentration of phenylglyoxal on the loss of enzymatic activity at 25°C. The phenylglyoxal reaction was carried out in 0.125 M potassium bicarbonate, pH 8.3, employing an enzyme concentration of 0.066 mg/ml. The enzymatic activity was determined by the colorimetric assay and is represented in semi-logarithmic form. The phenylglyoxal concentrations in the various runs were 28.1 mM (○), 14.1 mM (●), 7.0 mM (□), 4.7 mM (■) and 3.28 mM (△). Inset, variation of the pseudo-first-order rate constant (k) of the phenylglyoxal inactivation reaction with phenylglyoxal concentration. The rate constants were determined from the individual runs by fitting the experimental data to the first-order kinetic equation by non-linear least-squares.

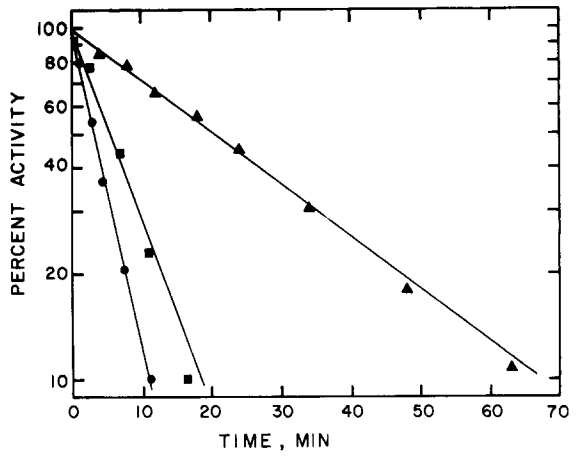

Fig. 2. The reaction of phenylglyoxal with ornithine transcarbamylase, native aspartate transcarbamylase and the catalytic subunit of aspartate transcarbamylase. The reactions were carried out at 25°C in 0.125 M potassium bicarbonate buffer pH 8.3 and a phenylglyoxal concentration of 0.7 mM. Percent activity, determined by the colorimetric assay, is shown versus time in a semi-logarithmic representation. Enzyme concentrations were 0.150 mg/ml for native aspartate transcarbamylase (●), 0.122 mg/ml for the catalytic subunit of aspartate transcarbamylase (■) and 0.066 mg/ml for ornithine transcarbamylase (▲).

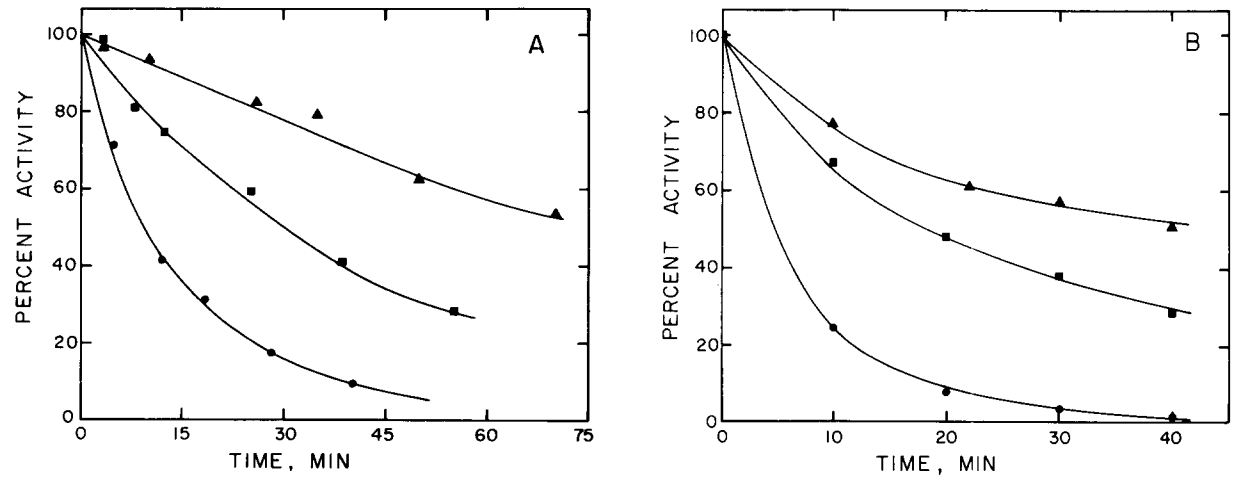

Fig. 3. Inactivation of ornithine transcarbamylase and the catalytic subunit of aspartate transcarbamylase in the presence and absence of substrates and substrate analogs. (A) Ornithine transcarbamylase at a concentration of 0.066 mg/ml was reacted with 14.1 mM phenylglyoxal in 0.125 M potassium bicarbonate buffer, pH 8.3. Inactivation was measured by the colorimetric assay and the data are presented as percent activity versus time. The reaction was carried out in the absence of ligands (●), in the presence of 10 mM carbamyl phosphate (■), and in the presence of 10 mM carbamyl phosphate plus 10 mM norvaline (▲). (B) The catalytic subunit of aspartate transcarbamylase at a concentration of 0.78 mg/ml and a phenylglyoxal concentration of 2.3 mM. The reaction rate was monitored by the loss of enzymatic activity by the pH-stat assay at pH 8.3, and the data are presented as percent activity versus time. The modification reaction was carried out in 0.1 M *N*-ethylmorpholine buffer, pH 8.3 at 25°C, in the presence of no ligands (●), 10.7 mM carbamyl phosphate (■) and 10.7 mM carbamyl phosphate plus 10.2 mM succinate (▲).

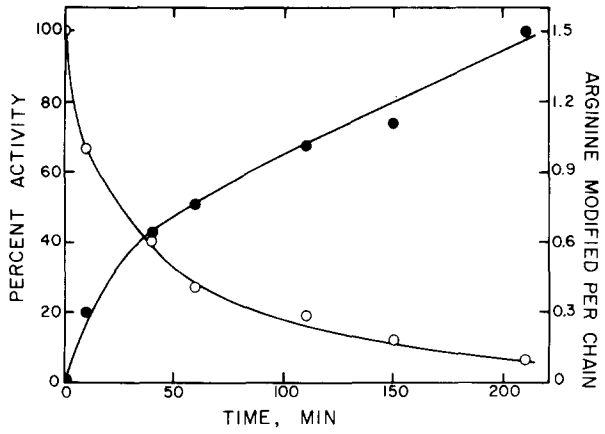

Fig. 4. Incorporation of radioactive phenylglyoxal into ornithine transcarbamylase. The number of arginine residues modified was determined by incorporation of [^{14}C]phenylglyoxal. The reaction was carried out at 25°C employing 1.09 mM [^{14}C]phenylglyoxal and 0.26 mg/ml ornithine transcarbamylase in 0.125 M potassium bicarbonate, pH 8.3. The loss of enzymatic activity was monitored by the colorimetric assay. The left ordinate describes the percent loss of activity relative to activity at time zero (○) and the right ordinate describes the number of modified arginine residues per chain as a function of time (●).

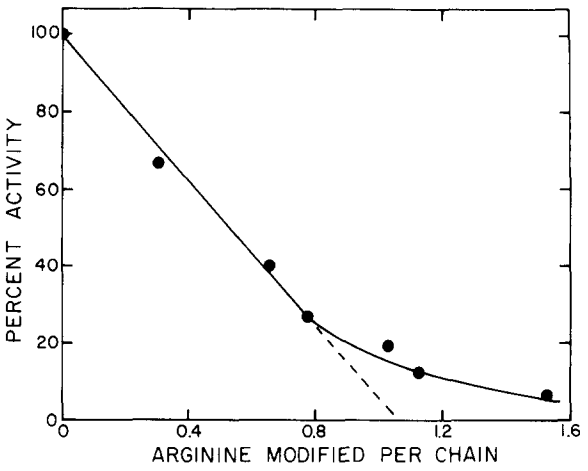

Fig. 5. Activity as a function of the number of arginine residues modified in ornithine transcarbamylase. The number of arginine residues modified is expressed per 35 000 molecular weight (one chain). The reaction conditions are the same as those reported in the legend to Fig. 4.

glyoxal was slowed by reducing the phenylglyoxal concentration. Aliquots were removed at timed intervals and the reaction quenched by Sephadex G-25 chromatography. As seen in Fig. 4, as the activity is diminished increasing numbers of arginine residues are modified. The data in Fig. 4 are plotted per 35 000 daltons, which corresponds to one of the enzyme's three polypeptide chains. The decrease in activity follows simple first-order kinetics; however, the incorporation of [^{14}C]phenylglyoxal is kinetically more complex. This would be expected if residues not necessary for activity are also reacting but at a somewhat slower rate. By directly correlating the incorporation of label with loss of activity, (see Fig. 5) the differential reaction rates can be clearly observed. Extrapolation of the nearly linear portion of this graph indicates that approx. one arginine per active site is essential to the enzyme's activity. Additional arginine residues not involved in the enzyme's catalysis also react, but at a significantly slower rate.

Discussion

The data in this paper support the conclusions that: there is an arginine residue at the active site of ornithine transcarbamylase; that this residue is necessary for the activity of the enzyme; and that the active site of ornithine transcarbamylase is similar to the active site of aspartate transcarbamylase.

For *E. coli* ornithine transcarbamylase, phenylglyoxal was found to inactivate the enzyme completely. Furthermore, loss of activity correlates with the modification of one arginine residue per active site. Although ornithine transcarbamylase contains a considerable number of arginine residues, phenylglyoxal reacts primarily with just one. Similar observations have been observed for many other enzymes such as ribonuclease [22], glutamine and carbamyl phosphate synthetase [23], alcohol dehydrogenase [24], phospholipase A_2 [25] and aspartate transcarbamylase [10]. The environment of the reactive arginine seems to be extremely important in respect to the relative rate of the phenylglyoxal inactivation.

In order to deduce the site of the modification in ornithine transcarbamylase, substrates and substrate analogs were used to determine if the rate of the inactivation reaction would be affected. Considerable

protection from inactivation was afforded the enzyme by carbamyl phosphate plus norvaline, an analog of ornithine. This protection from inactivation by molecules which bind at the enzyme's active site is a strong indication that the site of the essential arginine is at the active site. Since carbamyl phosphate alone can protect against inactivation by phenylglyoxal, it is reasonable to conclude that the arginine necessary for activity is close to the carbamyl phosphate binding site. The increased protection afforded by the combination of carbamyl phosphate and norvaline is the result of tighter binding of carbamyl phosphate when the analog is bound in the ornithine site. The protection experiment could not be performed with norvaline alone, because it does not bind without carbamyl phosphate.

The rate of phenylglyoxal inactivation of ornithine transcarbamylase is considerably slower than for aspartate transcarbamylase under identical conditions. However, the rate of inactivation of the catalytic subunit of aspartate transcarbamylase was slower than the native enzyme but faster than ornithine transcarbamylase. This suggests that as far as reactivity towards phenylglyoxal is concerned, ornithine transcarbamylase is more similar to the catalytic subunit of aspartate transcarbamylase than to the native enzyme.

Because of the similarities between ornithine transcarbamylase and the catalytic subunit of aspartate transcarbamylase, the protection experiments with substrates and substrate analogs were repeated with the catalytic subunit. Although there is a difference in the rate of phenylglyoxal inactivation for these two species the protection afforded by carbamyl phosphate was almost identical. The inactivation rate was slowed 3.2-times for ornithine transcarbamylase compared to 3.8-times for the catalytic subunit of aspartate transcarbamylase. The combination of carbamyl phosphate and the appropriate substrate analog, norvaline or succinate, slowed the rates even further. For ornithine transcarbamylase, the inactivation rate was slowed 8.2-times compared to 9.5-times for the catalytic subunit of aspartate transcarbamylase. As seen in Fig. 3, the protection by the substrate carbamyl phosphate or carbamyl phosphate plus a substrate analog is almost identical, further emphasizing the similarities that must exist in the active sites of these two enzymes. The almost identical behavior towards protection further supports the notion that the arginine is at the carbamyl phosphate binding site because this is the substrate which is common to the two enzymes.

Recently, it has been reported that butanedione and phenylglyoxal inactivate bovine ornithine transcarbamylase [26]. The results reported here imply that the active sites of prokaryotic and eukaryotic ornithine transcarbamylases both contain an arginine residue and that this residue is necessary for the reaction. The purpose of this arginine residue is most likely for the binding of the phosphate group of carbamyl phosphate. In a great many cases, arginine residues have been implied in binding negatively-charged substrates in general and phosphate groups in particular.

This study shows that the active sites of *E. coli* ornithine and aspartate transcarbamylase are similar, at least with respect to a single essential arginine residue. Furthermore, the similar reactivities of these two enzymes toward phenylglyoxal, under identical conditions indicate similarities in the geometry of the active site.

Other evidence is also available which suggests that these enzymes are related. For example, genetic studies have revealed that *pyrB*, the gene coding for aspartate transcarbamylase and *argI*, the gene coding for ornithine transcarbamylase are adjacent on the *E. coli* chromosome [27,28]. A more direct comparison of the similarities that exist between the two enzymes has come from a comparison of the sequence of their first 37 amino acid residues [29]. These data show that 27% of the residues are identical with another 11% possible. When the amino acid variations resulting from a single base change are considered, then 76% of the residues are identical. Another similarity between these transcarbamylases is their ability to be inhibited by corresponding transition state analogs, *N*-phosphonacetyl-L-aspartate being a potent inhibitor of aspartate transcarbamylase [30], and *N*-phosphonacetyl-L-ornithine being a potent inhibitor of ornithine transcarbamylase [31,32].

The close proximity of the ornithine and aspartate transcarbamylase genes on the *E. coli* chromosome [27,28] and the similarities in the quarternary structures of these two enzymes were the first indications that they may be evolutionarily related. With the comparison of their N-terminal amino acid sequences the

possibility of a relationship has become more obvious. Now with this report of the first chemical tests of relative reactivities between *E. coli* ornithine and aspartate transcarbamylase, the likelihood of an evolutionary relationship between them is even more evident. We are currently in the process of further characterizing the similarities that exist between these two enzymes.

Acknowledgements

A.F.F. is a recipient of a James Flack Norris Undergraduate Summer Research Scholarship administered by the Northeastern Section of the American Chemical Society. Acknowledgement is made to the Donors of the Petroleum Research Fund, administered by the American Chemical Society for the support of this research.

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