Site-Directed Mutagenesis of *Escherichia coli* Ornithine Transcarbamoylase: Role of Arginine-57 in Substrate Binding and Catalysis†,‡

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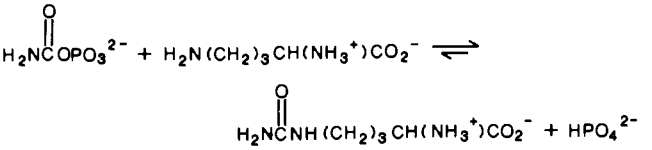
Received April 29, 1988; Revised Manuscript Received July 29, 1988

ABSTRACT: In the carbamoyl-transfer reaction catalyzed by ornithine transcarbamoylase, an arginine residue in the active site of the *Escherichia coli* enzyme has been suggested to bind the phosphate moiety of the substrate carbamoyl phosphate. With the application of site-specific mutagenesis, the most likely arginine residue among three candidates at the binding site of carbamoyl phosphate, Arg-57, has been replaced with a glycine. The resultant Gly-57 mutant enzyme is drastically inefficient in catalysis. In the synthesis of L-citrulline from carbamoyl phosphate and L-ornithine with the release of inorganic phosphate, the turnover rate of the mutant is 21 000-fold lower than that of the wild type. However, the mutation of Arg-57 affects only moderately the binding of carbamoyl phosphate; the dissociation constant of this substrate, measured under steady-state turnover condition, is increased from 0.046 to 3.2 mM by the mutation. On the other hand, ornithine binding is substantially affected as estimated by the change in the dissociation constant of its analogue L-norvaline. The dissociation constant of L-norvaline increases about 500-fold from 54 μM for the wild type to 25 mM for the mutant. Since Arg-57 is expected to be distal from the ornithine site and the amino acid (both ornithine and norvaline) binds only after carbamoyl phosphate in the wild-type reaction, the poor norvaline affinity to the mutant suggests that Arg-57 is involved in interactions essential for productive addition of the amino acid. This interpretation is supported by difference ultraviolet absorption spectra which show that the conformational changes induced in the wild type by carbamoyl phosphate upon binding are absent in the mutant. Furthermore, steady-state kinetic data reveal that the ordered binding mechanism of the wild-type enzyme is transformed into a random binding mechanism in the mutant. Thus, the presence of carbamoyl phosphate in the mutant active site is no longer a requisite for ornithine binding. In the 5–50 °C temperature range, transcarbamoylation catalyzed by either the wild type or the mutant observes the Arrhenius rate law with almost identical enthalpies of activation, 11 and 10 kcal/mol, respectively. The entropy of activation is −5.5 eu for the wild-type reaction and −29 eu for the mutant reaction, accounting for a loss of 6–7 kcal/mol in the rate-determining step of the enzymic reaction. The combined results indicate that the difference in catalytic efficiency between the two enzymes is entropic in origin—the Gly-57 mutant binds carbamoyl phosphate less strongly, it does not undergo conformational transitions to initiate proper stereospecific binding of ornithine, and it is incapable of promoting tight binding of the transition state associated with the rate-determining step of the reaction.

Ornithine transcarbamoylase (EC 2.1.3.3) catalyzes the synthesis of L-citrulline from carbamoyl phosphate and L-ornithine in the urea cycle. Because L-arginine is the penultimate product of the urea cycle, transcarbamoylation of ornithine in mammals is a step in the pathway of arginine biosynthesis. This function is highly conserved in evolution. Indeed, ornithine transcarbamoylase from the human liver (Horwich et al., 1984) resembles that from *Escherichia coli* (Legrain et al., 1972) both in subunit and in primary structures; both are trimeric molecules ~110 000 daltons in mass and composed of identical subunits.

Ornithine transcarbamoylase normally observes Michaelis–Menten kinetics; its binding of substrates and release of products are both sequential (Marshall & Cohen, 1972; Legrain & Stalon, 1976; Wargnies et al., 1978). In the forward reaction, carbamoyl phosphate is the first substrate bound, and inorganic phosphate is the second product released:



Most intriguingly, the enzymes from *E. coli* (Kuo et al., 1982; Kuo, 1983) and from the human liver (R. M. Emkey and L. Kuo, unpublished data) display sigmoidal kinetics in the presence of Zn^{2+}. This enzyme also demonstrates very high substrate specificity (Marshall & Cohen, 1972; Nakamura & Jones, 1970). Only carbamoyl phosphate and L-ornithine are good substrates of the enzyme. Activity of the enzyme obtained with acetyl phosphate as substrate is ~10% of that obtained with carbamoyl phosphate. L-Lysine and L-2,4-diaminobutyrate are even poorer substrates; they afford only 2–4% of the enzymic activity obtained with L-ornithine. In fact, only the minor zwitterion of L-ornithine with an uncharged δ-amino group, $H_2N(CH_2)_3CH(NH_3^+)CO_2^-$, binds the enzyme productively (Kuo et al., 1985). This stringent specificity raises the apparent K_M for ornithine but is ther-

† This research was supported by Grant DK38089 from the National Institute of Diabetes and Digestive and Kidney Diseases and in part by a Cottrell Research Grant from the Research Corp. L.C.K. is an NIH Research Career Development Awardee (Grant DK01721) and a Pew Scholar of the Pew Charitable Trusts.

‡ The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02842.

0006-2960/88/0427-8823$01.50/0

modynamically advantageous; it leads to the binding of an ornithine with an δ-NH_2 poised for nucleophilic attack.

Other mechanistic details of the transcarbamoyl reaction are scarce. The rate-determining step of the enzymic reaction is unknown. Knowledge of the active site environment of the enzyme remains limited to results from chemical modification studies. Residues suggested to be responsible for substrate binding include a lysine, a cysteine, and an arginine (Marshall & Cohen, 1977, 1980a,b; Fortin et al., 1981). X-ray structural studies on ornithine transcarbamoylase are in the rudimentary stage; two forms of diffracting single crystals of the enzyme have now been obtained (L. Kuo and B. Seaton, unpublished results). For the closely related enzyme aspartate transcarbamoylase, which catalyzes an analogous reaction in the pyrimidine biosynthetic pathway, recent crystallographic studies have provided a complete picture of the immediate vicinity of its active site (Volz et al., 1986; Krause et al., 1987). The amino acid sequences of the two transcarbamoylases are highly homologous (Schachman et al., 1984; Bencini et al., 1983). Of the 12 amino acids involved in binding the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate to *E. coli* aspartate transcarbamoylase, 10 are found to be conserved in *E. coli* ornithine transcarbamoylase.[1] The two enzymes are so similar that when the carbamoyl phosphate domain of ornithine transcarbamoylase (*argI*) is joined to the aspartate domain of aspartate transcarbamoylase (*pyrB*), an active aspartate carbamoyltransferase is obtained (Houghton, 1986). The carbamoyl phosphate domain of these two enzymes is apparently interchangeable. Thus despite the lack of X-ray diffraction data at present on ornithine transcarbamoylase, it is possible to probe the role of specific active-site residues of the bacterial enzyme.

We have begun our study on the carbamoyl phosphate binding site of *E. coli* ornithine transcarbamoylase with the application of site-directed mutagenesis. Our initial focus is directed toward the arginine residues. Arg-57 and Arg-106 are implicated in forming ionic bonds with oxygen atoms of the phosphate group of carbamoyl phosphate.[1] Arg-59 has not been shown to be involved in the "transition state" of the reaction, but its proximity to Arg-57 warrants its inclusion in our study, and this residue may be important in the initial stage of substrate binding or in product release. In this report, we present our finding on the mutation at the Arg-57 site. This residue is chosen to be the first target because in a number of phosphate binding proteins the phosphoryl site has been shown (Schulz & Schirmer, 1979; Sachsenheimer & Schulz, 1977) to contain a hydroxyl residue, usually a serine, separated by a single amino acid from an arginine or a lysine.[2] This stretch of sequence is also found in the carbamoyl phosphate sites of ornithine transcarbamoylase (Ser^{55}-Thr^{56}-Arg^{57}-Thr^{58}) and aspartate transcarbamoylase (Ser^{52}-Thr^{53}-Arg^{54}-Thr^{55}). For the latter enzyme, all four residues are involved in binding the phosphate moiety of *N*-(phosphonoacetyl)-L-aspartate (Volz et al., 1986; Krause et al., 1987).

We have decided to replace Arg-57 with glycine in order to incorporate the most drastic changes in terms of both charge and bulk. Our rationale is that for residues that are *not* essential for binding and catalysis such changes should alter only slightly or not at all the catalytic efficiency of the enzyme. Replacement of the arginine side chains in the carbamoyl phosphate site with glycine allows us to assess their importance in binding and catalysis. The next logical approach will be to replace those that are essential for catalysis with histidine and with glutamine (or isoleucine) in order to assess the specific role of charge and bulkiness of these residues.

Materials and Methods

Materials. The oligonucleotides used for site-directed mutagenesis and DNA sequencing were synthesized with use of a Beckman System-1-Plus DNA synthesizer and a Milligen 6500 DNA synthesizer. The oligonucleotides prepared with the Beckman machine were purified by reverse-phase HPLC utilizing a Beckman C3 Ultrapore column. Oligonucleotides prepared with the Milligen synthesizer were used without further purification. Sequencing reagents, restriction endonucleases, and other enzymes were purchased from New England Biolabs and used according to the supplier's recommendations. Radioactive α-[^{35}S]thiodideoxy-ATP was acquired from New England Nucleotides. A 10.8-kb plasmid derivative of pBR322 containing *argI*, pAI101 (Bencini et al., 1983), was a gift from Professor James Wild of Texas A&M University. The *E. coli* strains JM101, TG1 (*Δpro–lac,-supE,thi,hsdD5*/F'*traD36,proA*$^+$*B*$^+$,*lacI*^{q},*lacZΔM15*) (Carter et al., 1985), HB2154 (*ara,Δpro–lac,thi*/F'*proA*$^+$*B*$^+$,*lacI*^{q},-*lacZΔM15*,mutL::Tn10) (Carter et al., 1985), and TB2 (*ΔargI–pyrB,argF*$^-$) (Bencini et al., 1983) were obtained from Professor Evan Kantrowitz of Boston College.

Cloning of argI into M13mp19. The cloning of the *argI* gene was conducted by employing methods of Maniatus et al. (1982) as described below unless noted otherwise. The *argI* gene fragment from pAI101 was first cloned into the polylinker region of pUC18 to create a 7.5-kb subclone prior to its cloning into the vector M13mp19. A *Hpa*I restriction digest of pAI101 produced two DNA fragments, ~3.4 and ~7.3 kb in size, which were then treated with *Bal*31 for 2.5 min at 30 °C without prior separation of the fragments. This treatment produced two sets of heterogeneous DNA fragments of ~2.1 and ~6 kb. The reaction mixture was run on a 1% agarose gel, and the 2.1-kb DNA fragments were retrieved from the gel with use of the NaI–glass powder procedure (Vogelstein & Gillespie, 1979). The DNA obtained was then treated with mung bean nuclease and ligated with T4 DNA ligase into the *Hin*cII site of the polylinker region of pUC18. The product of the ligation reaction was then transformed (Davis et al., 1986) into competent TB2 cells. Colonies were selected from M9 plates containing 30 μg/mL uracil and 50 μg/mL ampicillin. Since TB2 cells contained no chromosomal *argI* and *argF* genes and could not synthesize L-arginine, the minimal M9 media was employed to select for a functional *argI* insert

[1] The residues of aspartate transcarbamoylase involved are Ser-52, Thr-53, Arg-54, Thr-55, Ser-80, Lys-84, Arg-105, and His-134 in the binding domain of carbamoyl phosphate and Arg-167, Arg-229, Gln-231, and Leu-267 in the binding domain of aspartate (Volz et al., 1986; Krause et al., 1987). The corresponding residues of ornithine transcarbamoylase are Ser-55, Thr-56, Arg-57, Thr-58, Ser-81, Lys-86, Arg-106, His-133, Arg-165, and Leu-274. Of the eight residues in the binding domain of carbamoyl phosphate, homology is implicated for all in the primary sequence as well as in the secondary structure of the enzymes (Houghton et al., 1984). Of the four residues implicated in the amino acid site of aspartate transcarbamoylase, only two are found in the primary sequence of ornithine transcarbamoylase, and neither is homologous in the secondary structure of the enzymes. Since L-aspartate is a monoamino acid while L-ornithine is a diamino acid, it is not surprising to find virtually no homology at the binding sites of these substrates. Further, it should be noted that the phosphate moiety of carbamoyl phosphate is displaced by the α-amino group of L-aspartate to form *N*-carbamoyl-L-aspartate in the reaction of aspartate transcarbamoylase but by the δ-amino group of L-ornithine to form L-citrulline in the reaction of ornithine transcarbamoylase.

[2] These phosphoryl binding proteins include the myelin protein, histone H1, histone H2, histone H4, troponin I, flavodoxin, glycogen synthetase, and adenylate kinase.

while ampicillin was employed to select for the pUC18 fragment. (TB2 cells also contained no *pyrBI* gene and required uracil for growth). Plasmids were isolated from six colonies by the modified alkaline lysis method as described by Maniatus et al. (1982) and analyzed by means of restriction digest with *Eco*RI and *Hin*dIII. The desired plasmid, G1pUC18wt, contained *argI* in a 2.1-kb fragment inserted within the polylinker region of pUC18. This 2.1-kb fragment was then conveniently cloned into the *Eco*RI–*Hin*dIII site of M13mp19; the resultant vector, G1mp19wt, was used for DNA sequencing and site-directed mutagenesis.

Mutagenesis Procedure. Site-directed mutagenesis was accomplished by following the procedure of Zoller and Smith (1982) as modified by Norris et al. (1983). A 21-base oligonucleotide was synthesized to introduce the specific C → G base change necessary for the substitution of a glycine for the wild-type arginine at residue 57 of ornithine transcarbamoylase. To ensure that no secondary priming sites existed for this mutant oligonucleotide, the sequence of the oligonucleotide was first matched against the entire sequence of the *argI* operon. Only one priming site was found. The oligonucleotide was then used as a primer in Sanger sequencing (Sanger et al., 1977). Clear and unambiguous sequencing was obtained, indicating that this oligonucleotide did indeed anneal to only the desired site. The mutant oligonucleotide was phosphorylated and annealed to a single-strand G1mp19wt template in a single-primer reaction. The annealed oligonucleotide was then extended with dNTPs and Klenow and transformed into competent HB2154 cells with the standard $CaCl_2$/heat-shock procedure (Davis et al., 1986). The transformed cells were plated on LB lawns containing TG1 cells for plaque selection. Ten candidate plaques were picked for isolation of single-stranded DNA and sequenced with the Sanger technique. Four of the ten candidates were found to contain the C → G single mutation at the desired site. The *Eco*RI–*Hin*dIII insert from one of the mutants was cloned into pUC18, and the resultant plasmid, G1pUC18m2, was transformed into TB2 cells. Finally, to ensure that only Arg-57 was modified, the *Eco*RI–*Hin*dIII insert from G1pUC18m2 was recloned into M13mp19 and the entire *argI* gene sequenced. This step was necessary because in the selection of point mutant the host cells (HB2154) used were deficient in DNA repair mechanism (Carter et al., 1985) and extraneous mutations could have occurred. Only the single C → G mutation was found at the designated site in *argI*.

Cell Growth. Both the wild-type and mutant ornithine transcarbamoylases were expressed in the host strain TB2. TB2 cells containing either the wild-type (G1pUC18wt) plasmid or the mutant (G1pUC18m2) plasmid were grown at 37 °C in LB broth (Schleif & Wensink, 1984) in the presence of 50 μg/mL ampicillin for 12 h until exponential growth phase was reached. The bacterial cells were harvested by centrifugation at 4 °C.

Enzyme Purification. Both the wild-type enzyme and the Gly-57 mutant were purified from 10-L cultures of TB2 cells following the procedure described by Kuo et al. (1982). However, the chromatographic columns used previously, a DEAE-Sephadex A-50 column, an Ultrogel column, and an AH-Sepharose 4B column, were substituted by a single Matrex Blue gel column. Following the ammonium sulfate precipitation steps, the proteins obtained were dissolved and dialyzed in 20 mM Tris–acetate, pH 7.5, and chromatographed at ambient temperature on a Matrex Blue gel column that had been preequilibrated with the same buffer. For the mutant, the enzyme was eluted with 20 mM Tris–acetate buffer, pH 7.5. For the wild-type enzyme, the column was first washed with 20 mM Tris–acetate, pH 7.5, until protein absorbance at 280 nm was null; the enzyme was then eluted with a linear gradient of 0–0.5 M KCl in 20 mM Tris–acetate buffer, pH 7.5. Enzyme homogeneity for both enzymes was indicated by a single band on $NaDodSO_4$ polyacrylamide gels stained with Coomassie Blue. The extinction coefficient for both enzymes at 280 nm was determined to be 0.96 OD per mg/mL of protein per centimeter of path length.

Enzyme Assays. Steady-state initial velocity assays were performed as described previously (Kuo et al., 1985). Initial velocity was taken to be the rate of L-citrulline production as determined by use of the method of Pastra-Landis (1981). The quantity of enzyme used was kept low in all assays so that a sufficiently high ratio of [S]/[E] was maintained to ensure steady-state turnover. Under all circumstances, the rates collected were initial velocities. Assay temperature was thermostated to within ±1 °C, and the pH of reaction buffers was adjusted at the assay temperature. Other details are given in the figure legends.

Kinetic Data Analysis. For comparison of k_{cat} and apparent K_M (K_M^{app}) values in the 5–50 °C range, initial velocity data were fit to the Michaelis–Menten equation by nonlinear regression methods. Enthalpies of activation (ΔH_{act}) were obtained from the slope of Arrhenius plots, ln (k/T) versus $1/T$. Entropies of activation (ΔS_{act}) were calculated from eq 1,

$$k = (K_B T/h) \exp(-\Delta H_{act}/RT) \exp(\Delta S_{act}/R) \quad (1)$$

where k is a rate constant, K_B is Boltzmann's constant, T is the temperature, h is Planck's constant, and R is the gas constant.

For dead-end inhibition studies, double-reciprocal plots and slope replots were made to determine kinetic patterns. Data were then fit by weighted nonlinear regression to the appropriate form of eq 2 as described below. The nonreciprocal form of eq 2 was used for fitting. Weights were based on constant relative error in velocity in conjunction with bisquare reweighting to reduce the influence of outliers (Holland & Welsch, 1977). The values given in Tables I and III represent the average of values obtained from several experiments.

$$v_0^{-1} = V_{max}^{-1}(1 + K_a/A + K_b/B + K_{ia}K_b/AB) \quad (2)$$

The nomenclature of Cleland is followed. The K_a and K_b are the Michaelis constants of carbamoyl phosphate and L-ornithine, K_{ia} is the dissociation constant of carbamoyl phosphate from its binary complex with the enzyme, and A and B are respectively the concentrations of carbamoyl phosphate and ornithine. In the case of a rapid-equilibrium random mechanism, the Michaelis constants are also the dissociation constants of the substrates from the *ternary* enzyme–substrate complex, and the dissociation constant of ornithine from the enzyme–ornithine binary complex is equal to K_bK_{ia}/K_a. Where substrate inhibition is observed, an additional term, B/K_{Ib}, is added to the sum given in the parentheses in eq 2.

In the presence of a competitive inhibitor (I), two dissociation constants can be defined: K_i, for the binding of inhibitor to free enzyme, and K_{ii}, for its binding to the complex between the enzyme and the substrate with which the inhibitor does *not* compete. For the ordered Bi Bi mechanism, the correct form of eq 2 is one in which both the K_a/A and $K_{ia}K_b/AB$ terms are multiplied by $(1 + I/K_i)$ when a competitive inhibitor of A is present while the K_b/B term is multiplied by $(1 + I/K_{ii})$ when a competitive inhibitor of B is present. For the rapid equilibrium random mechanism, the K_a/A (or K_b/B) term is multiplied by $(1 + I/K_{ii})$ and the K_iK_b/AB term is multiplied

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TCC GGG TTT TAT CAT AAG CAT TTC CTG AAA TTA CTC GAT TTC ACG CCA GCT GAA CTC AAC AGC CTG CTG CAG TTA
ser gly phe tyr his lys his phe leu lys leu leu asp phe thr pro ala glu leu asn ser leu leu gln leu
1                                                                                              25

GCC GCG AAG CTG AAA GCC GAT AAG AAA AGC GGT AAA GAA GAA GCC AAA CTC ACT GGT AAA AAC ATC GCG CTC ATC
ala ala lys leu lys ala asp lys lys ser gly lys glu glu ala lys leu thr gly lys asn ile ala leu ile
26                                                                                             50

TTC GAA AAA GAC TCG ACT CGT ACC CGA TGC TCT TTC GAA GTT GCC GCA TAT GAC CAG GGT GCT CGC GTT ACT TAT
phe glu lys asp ser thr arg thr arg cys ser phe glu val ala ala tyr asp gln gly ala arg val thr tyr
51                                                                                             75

CTC GGC CCA AGC GGC AGC CAG ATT GGT CAT AAA GAG TCG ATT AAA GAC ACT GCC CGC GTG CTT GGT CGC ATG TAT
leu gly pro ser gly ser gln ile gly his lys glu ser ile lys asp thr ala arg val leu gly arg met tyr
76                                                                                            100
                                                                          *       **
GAC GGT ATT CAG TAT CGC GGC TAT GGT CAG GAG ATT GTC GAA ACA CTG GCG GAA TAC GCT AGC GTG CCG GTA TGG
asp gly ile gln tyr arg gly tyr gly gln glu ile val glu thr leu ala glu tyr ala ser val pro val trp
101                                                                                           125
                                                 * *  *  •   ***
AAT GGC CTG ACC AAT GAG TTC CAT CCC ACG CAG CTG CTG GCG GAT CTT CTC ACC ATG CAG GAG CAT TTG CCC GGC
asn gly leu thr asn glu phe his pro thr gln leu leu ala asp leu leu thr met gln glu his leu pro gly
126                                                                                           150

AAA GCG TTC AAC GAA ATG ACG CTG GTC TAT GCA GGT GAC GCG CGT AAC AAC ATG GGC AAT TCG ATG CTC GAA GCT
lys ala phe asn glu met thr leu val tyr ala gly asp ala arg asn asn met gly asn ser met leu glu ala
151                                                                                           175

GCG GCG CTT ACC GGT CTG GAT TTG CGT CTG GTC GCG CCA CAA GCG TGC TGG CCG GAA GCT GCG CTG GTT ACG GAA
ala ala leu thr gly leu asp leu arg leu val ala pro gln ala cys trp pro glu ala ala leu val thr glu
176                                                                                           200

TGC CGC GCC CTG GCA CAG CAA AAT GGT GGG AAT ATT ACG CTG ACT GAA GAT GTC GCG AAG GGA GTT GAA GGT GCT
cys arg ala leu ala gln gln asn gly gly asn ile thr leu thr glu asp val ala lys gly val glu gly ala
201                                                                                           225
                                                          *
GAC TTT ATC TAT ACC GAT GTG TGG GTG TCG ATG GGG GAA GCA AAA GAG AAA TGG GCG GAA CGG ATT GCA TTG CTG
asp phe ile tyr thr asp val trp val ser met gly glu ala lys glu lys trp ala glu arg ile ala leu leu
226                                                                                           250
**
CGT GAA TAT CAG GTG AAC AGC AAG ATG ATG CAG TTG ACC GGT AAC CCG GAG GTC AAA TTC CTC CAC TGC CTG CCC
arg glu tyr gln val asn ser lys met met gln leu thr gly asn pro glu val lys phe leu his cys leu pro
251                                                                                           275

GCG TTT CAT GAC GAC CAA ACG ACG CTT GGC AAG AAA ATG GCG GAA GAA TTT GGC CTA CAT GGC GGT ATG GAA GTC
ala phe his asp asp gln thr thr leu gly lys lys met ala glu glu phe gly leu his gly gly met glu val
276                                                                                           300
                                                    *
ACT GAT GAG GTC TTC GAA TCT GCC GCC AGC ATT GTT TTT GAT CAG GCG GAA AAC CGT ATG CAT ACT ATC AAA GCG
thr asp glu val phe glu ser ala ala ser ile val phe asp gln ala glu asn arg met his thr ile lys ala
301                                                                                           325

GTG ATG GTC GCG ACG CTC AGT AAA TAA
val met val ala thr leu ser lys end
326                         333
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FIGURE 1: DNA sequence of *argI* of *E. coli* K12 and the deduced amino acid sequence of the ornithine transcarbamoylase polypeptide. Asterisks denote nucleotides that differ from those of the sequence reported by Bencini et al. (1983). The molecular weight of the trimer based on the sequence in this figure is 110 328.

by $(1 + I/K_i)$ in the presence of a competitive inhibitor of A (or B). The reader is referred to Fromm (1979) for a complete exposition.

Difference Ultraviolet and Circular Dichroism Spectra. Ultraviolet absorption spectra were recorded digitally on a Beckman DU-7 single-beam spectrophotometer with a spectral bandwidth of 2 nm. Buffered enzyme solutions were diluted with a small volume of water, buffer, or aqueous substrate/analogue solution, thermostated at 25 °C in a quartz cell of 10-mm path length, and scanned within a 10-min period. All spectra were recorded in triplicate and averaged. Difference spectra were calculated by subtracting digitally the absorbance of the free enzyme and free substrate/analogue from the spectrum of enzyme bound with carbamoyl phosphate and/or norvaline.

Circular dichroic absorbance of the wild-type and mutant enzymes was recorded on a Cary Model 61 spectropolarimeter that had been calibrated over the 190–500-nm wavelength region. Spectra of enzymic solutions, in 10 mM Tris–acetate buffer, pH 8.5, were recorded in triplicate from 205 to 250 nm in 1.0-mm quartz cuvettes at a full range of 0.05° and a protein concentration of 0.142 mg/mL for the wild type and 0.315 mg/mL for the mutant. Sample temperature was maintained at 25°C for all scans. Each spectrum was corrected for base line by subtracting the spectral contribution of the buffer solution.

RESULTS

DNA Sequence of argI. The complete DNA sequence of the wild-type *argI* of *E. coli* K12 is shown in Figure 1. This sequence differs from that reported by Bencini et al. (1983) at 14 positions as marked by asterisks. Our deduced amino acid sequence of the ornithine transcarbamoylase polypeptide is also shown in this figure. On the basis of our sequence, amino acid residues 118, 120, 138, 139, 140, 141, 241, 251, and 314 are respectively Glu, Ala, Leu, Ala, Asp, Leu, Glu, Arg, and Asp. The corresponding residues reported by Bencini et al. (1983) are Gln, Arg, Ile, Glu, Tyr, Lys, Gln, Ala, and Gly. On the basis of the amino acid sequence in Figure 1, the molecular weight of the trimeric enzyme is 110 328.

Table I: Comparison of k_{cat} and K_M^{app} of the Reaction Catalyzed by the Wild-Type (Arg-57) and Mutant (Gly-57) Ornithine Transcarbamoylases at pH 8.5 and 25 °C[a]

saturation	enzyme	k_{cat} (min^{-1})	K_M^{app} (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
L-ornithine	Arg-57 WT[b]	1.4 (0.02) × 10^5	0.32 (0.01)	0.44 (0.01) × 10^6
	Gly-57 mutant	6.6 (0.12)	0.31 (0.03)	21.3 (0.09)
carbamoyl phosphate	Arg-57 WT	1.3 (0.01) × 10^5	0.05 (0.001)	2.6 (0.3) × 10^6
	Gly-57 mutant	4.8 (0.13)	0.26 (0.01)	19 (1.1)

[a] In the ornithine saturation assays, carbamoyl phosphate concentration was kept at 5 mM; in the carbamoyl phosphate saturation assays, the ornithine concentration used was 3 mM. ±SE is given in parentheses. [b] WT, wild type.

Kinetic Parameters at 25 °C. A previous kinetic study on ornithine transcarbamoylase shows that the k_{cat}/K_M^{app} of this enzyme reaches a maximum at pH 7.5 and that k_{cat} reaches a plateau at pH ≥8.5 (Kuo et al., 1985). Thus, the kinetic properties of the wild type and mutant may be compared at either pH ~7.5 or pH ~8.5. We have chosen to conduct our assays at the higher pH because preliminary results of the steady-state activity of the mutant at different pHs reveal that its maximal turnover is in the pH 8–9 region.

Steady-state saturation studies for both enzymes have been conducted with either carbamoyl phosphate or L-ornithine as the varied substrate in the 5–50 °C range. In the ornithine assays, the maximum concentration of carbamoyl phosphate used, 5 mM, is at least 20-fold K_a^{app} and is sufficient to saturate 95–99% of the enzyme. No substrate inhibition by carbamoyl phosphate is observed for either enzyme. When carbamoyl phosphate is the varied substrate, the level of L-ornithine is maintained at ≤3 mM for the wild type because substrate inhibition is evident at ornithine concentrations greater than 4 mM. Surprisingly, no ornithine inhibition is observed for the mutant enzyme at concentrations of ornithine as high as 20 mM. This observation is discussed below.

The steady-state kinetic parameters, k_{cat} and K_M^{app}, for both the wild-type and the mutant enzymes at 25 °C are presented in Table I. The Gly-57 mutant is a very poor enzyme with a turnover rate of ~7 min^{-1}. This rate is 21 000-fold lower than that of the wild type. On the other hand, the K_a^{app} for the mutant (0.26 mM) is only 5-fold greater than that for the wild type (0.05 mM), and K_b^{app} for the two enzymes are the same (0.32 mM).[3] These K_M^{app} values suggest that insofar as the substrates saturating the enzyme for turnover is concerned there is no significant difference between the wild-type and mutant enzymes. Although K_M^{app} is often treated as an apparent dissociation constant, it is an unreliable gauge of binding affinity (see Discussion).

Substrate Binding Order. Before determining the dissociation and inhibition constants for comparison of binding affinities, the binding order of the substrates to the enzyme must be shown first so that the correct form of eq 2 may be applied.

For the wild-type enzyme from *E. coli* W and bovine liver, transcarbamoylation at pH 8–9 has been shown to observe the ordered Bi Bi mechanism with carbamoyl phosphate being the first substrate bound and citrulline the first product released (Marshall & Cohen, 1972; Legrain & Stalon, 1976; Wargnies et al., 1978). The same mechanism is confirmed for the wild-type enzyme from *E. coli* K12; norvaline is competitive against L-ornithine but uncompetitive against carbamoyl phosphate (Kuo et al., 1985). The uncompetitive inhibition pattern of norvaline against carbamoyl phosphate is diagnostic of an ordered mechanism with carbamoyl phosphate being the obligate first substrate bound. Further, the mechanism for the wild type is steady-state ordered and not rapid-equilibrium ordered. When L-ornithine is the varied substrate, the reciprocal plots for both enzymes at different concentrations of carbamoyl phosphate are linear and converge to the left of the $1/v_0$ ordinate, where v_0 is the initial velocity (data not shown).

[3] We have shown previously that only one zwitterionic species of L-ornithine is the true substrate of the enzyme (Kuo et al., 1985). The values of K_b^{app} reported in this paper are calculated for the entire ornithine population and are not those of the zwitterionic species.

Table II: Inhibition Patterns of L-Norvaline and Inorganic Phosphate in the Transcarbamoyl Reaction Catalyzed by Ornithine Transcarbamoylase

analogue	enzyme	type of inhibition[a]
L-norvaline	Arg-57 WT[b]	u (cp), c (orn)
	Gly-57 mutant	n (cp), c (orn)
inorganic phosphate	Arg-57 WT	c (cp), n (orn)
	Gly-57 mutant	c (cp), n (orn)

[a] c, competitive; n, noncompetitive; u, uncompetitive; cp, carbamoyl phosphate; orn, L-ornithine. [b] WT, wild type.

Table III: Dissociation Constants of Substrates and Analogues for Wild-Type (Arg-57) and Mutant (Gly-57) Ornithine Transcarbamoylase[a]

enzyme	reaction[b]	constant	mM
Arg-57 WT	E + cp = E·cp	K_{ia}	0.046 ± 0.016
	E + p_i = E·p_i	K_i	0.13 ± 0.01
	E·cp + orn = E·cp·orn	K_b	(0.00037)[c]
	E·cp + nor = E·cp·nor	K_{ii}	0.0089
Gly-57 mutant	E + cp = E·cp	K_{ia}	3.2 ± 0.3
	E + p_i = E·p_i	K_i	5 ± 0.9
	E + orn = E·orn	K_bK_{ia}/K_a	2.3 ± 0.3
	E + nor = E·nor	K_i	64 ± 4
	E·cp + orn = E·cp·orn	K_b	0.17 ± 0.01
	E·cp + nor = E·cp·nor	K_{ii}	25 ± 1
	E·orn + cp = E·cp·orn	K_a	0.24 ± 0.01
	E·orn + p_i = E · p_i·orn	K_{ii}	0.05 ± 0.03

[a] All assays were conducted at pH 8.5 and 25 °C under steady-state conditions as described under Materials and Methods. [b] cp, carbamoyl phosphate; nor, L-norvaline; orn, L-ornithine; p_i, inorganic phosphate. [c] Estimated using the ratio of norvaline affinities to the wild type and mutant (see Discussion).

For the mutant, norvaline remains competitive against ornithine but turns *noncompetitive* against carbamoyl phosphate. The altered inhibition pattern of norvaline is consistent only with a random rather than ordered binding mechanism. Thus, either substrate can bind the mutant enzyme first. This finding explains our observation that ornithine inhibition is absent in the mutant since for random addition substrate inhibition does not usually occur unless one substrate has affinity for the binding site of the other (Cleland, 1979).

Inorganic phosphate, as expected, is a competitive inhibitor of carbamoyl phosphate and a noncompetitive inhibitor of ornithine for either enzyme. These patterns are consistent with both ordered and random mechanisms. The inhibition patterns of L-norvaline and inorganic phosphate are listed in Table II.

Dissociation and Inhibition Constants. In Table III are listed the dissociation constants of substrates and analogues for both enzymes measured by steady-state kinetic assays. The

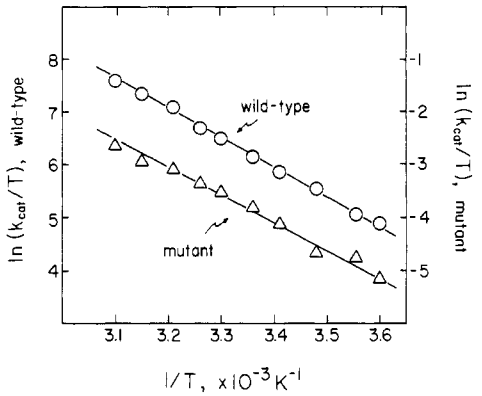

FIGURE 2: Comparison of the temperature dependence of the turnover rate constant, k_{cat}, for the wild-type (○) and mutant (△) reactions of *E. coli* ornithine transcarbamoylase. L-Ornithine saturation assays were performed at pH 8.5 in 1-mL solutions of 50 mM Tris–acetate buffer containing 5 mM carbamoyl phosphate and 5.2 ng of wild-type (or 110 μg of mutant) enzyme. At each temperature, the reaction time was adjusted so that initial-velocity condition was maintained (Kuo et al., 1985). The standard errors are smaller than the size of the symbol used.

affinity of carbamoyl phosphate is lowered 70-fold since K_{ia} is increased from 0.046 to 3.2 mM by the mutation.

The binding constant of L-ornithine can be calculated from initial velocity data for the random binding mechanism but not for the ordered mechanism, so we compare the binding affinity of L-norvaline instead. Since inhibition constants of dead-end inhibitor complexes can be measured directly under steady-state conditions and are true dissociation constants, this parameter is an accurate gauge of the binding strength between an enzyme and analogue in the presence of substrates. In the random mechanism there are two dissociation constants for norvaline. One is its binding constant to the free enzyme (K_i), and the other its binding constant to the enzyme–carbamoyl phosphate complex (K_{ii}). These dissociation constants can be obtained from saturation curves of the noncompetitive substrate at fixed concentrations of the analogue and the competitive substrate. Unexpectedly, the dissociation constant of L-norvaline from the ternary complex increases from 54 μM to 25 mM when the Arg-57 side chain is excised from the active site.

Table IV: Enthalpy and Entropy of Activation of the Ornithine Transcarbamoylase Reaction in the Forward Direction[a]

saturation		ΔH_{act} (kcal/mol)	ΔS_{act} (eu)
L-ornithine	Arg-57 WT	11.2 (0.3)	−5.5 (0.8)
	Gly-57 mutant	10.1 (0.3)	−29.3 (1.2)
carbamoyl phosphate	Arg-57 WT	11.3 (0.7)	−5.6 (2.3)
	Gly-57 mutant	10.0 (0.8)	−30.5 (2.6)

[a] Using 1 M as standard state for L-ornithine and carbamoyl phosphate. ±SE is given in parentheses.

Inorganic phosphate binds ~40-fold weaker to the free mutant than to the free wild-type enzyme. The dissociation constants of phosphate from the wild type and mutant are 0.13 mM and 5 mM, respectively.

Temperature Effect. The values of log (k_{cat}/T), obtained from ornithine saturation assays in the 5–50 °C range, are plotted against $1/T$ in Figure 2. Two conclusions may be drawn from this diagram. First, k_{cat} of the wild type remains 10^4-fold greater than that of the mutant throughout the entire temperature range tested. Second, the rate law of the transcarbamoyl reaction catalyzed by both enzymes may be described adequately by the Arrhenius relationship (eq 1). The ΔH_{act} of the two enzymic reactions are nearly identical at 11 ± 0.3 and 10 ± 0.3 kcal/mol for the wild-type and the mutant reactions, respectively. Therefore, the drop in turnover rate of the reaction as a result of the mutation is *entropic* in origin. Substituting these values into eq 1 yields a ΔS_{act} of −5.5 ± 0.8 eu for the wild type and and −29.3 ± 1.2 eu for the mutant. The same ΔH_{act} and ΔS_{act} values are obtained with kinetic parameters from carbamoyl phosphate saturation curves (Table IV).

In Figure 3 are shown the temperature profiles of K_a^{app} and K_b^{app}. From 5 to 50 °C, the temperature dependence of K_M^{app} of the mutant observes a linear relationship in the log K_M^{app} versus $1/T$ plot. In contrast, a distinct break is seen at 20 °C in both plots of the wild-type enzyme. Thus, both K_a^{app} and K_b^{app} of the mutant are controlled by one rate constant from 5 to 50 °C while those of the wild type are dominated by the rate constant of a single reaction step only in the 20–50 °C range. The linearity seen in the log K_M^{app} plots of the mutant is in accord with rapid equilibrium substrate binding, for which

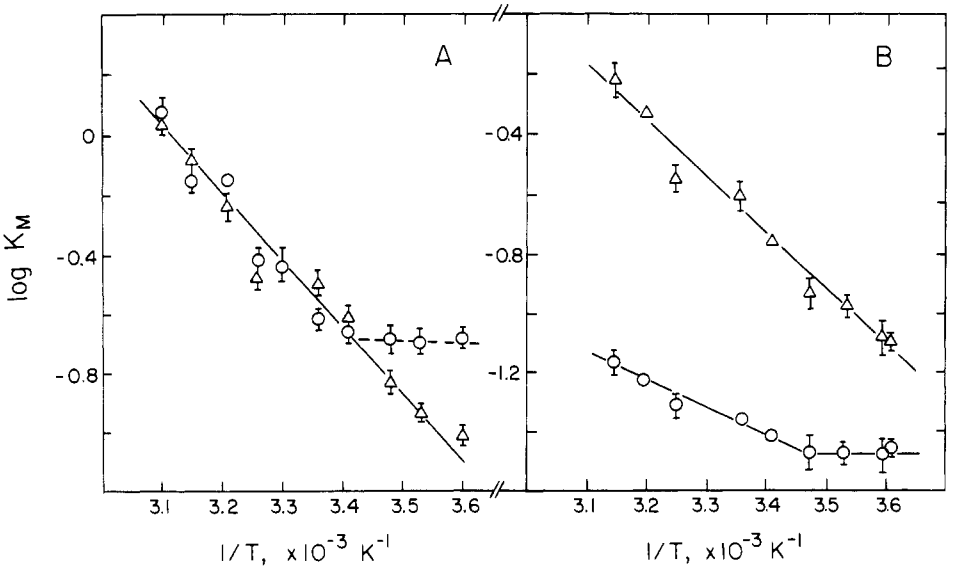

FIGURE 3: Comparison of the temperature dependence of the K_M^{app} of (A) L-ornithine and (B) carbamoyl phosphate for the wild-type (○) and mutant (△) reactions of ornithine transcarbamoylase. All conditions are as stated in the legend of Figure 2 and footnote *a* of Table I. Error bars indicate that standard error where it is larger than the size of the symbol used.

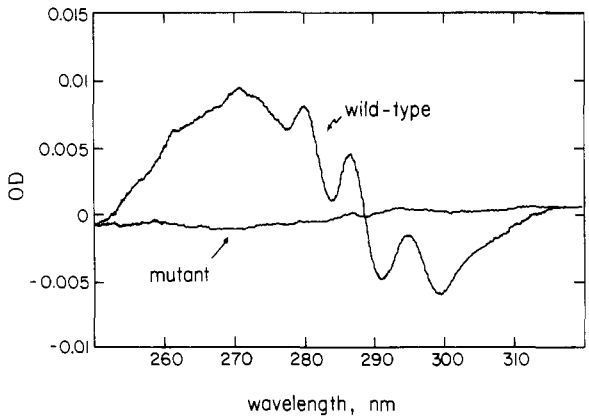

FIGURE 4: Ultraviolet difference spectra of wild-type and mutant ornithine transcarbamoylase in the presence of 0.05 M Tris–acetate and 2 mM carbamoyl phosphate, pH 8.5. The enzyme concentration was 1.1 mg/mL for the wild type and 0.6 mg/mL for the mutant. To facilitate comparison, the difference spectra have been normalized to give $A_{280} = 1.0$. The extinction coefficient of the wild type at 280 nm is 0.96 OD per mg/mL per cm of path length, from amino acid composition and dry-weight determinations, and is identical with that of the mutant.

K_a^{app} ($=K_a$) and K_b^{app} (K_b) are simple dissociation constants.

Above 20 °C K_b^{app} are the same for the two enzymes, while below 20 °C K_b^{app} of the mutant is *smaller* than that of the wild type. On the other hand, K_a^{app} of the mutant is 2.5–9 times greater than that of the wild-type over the entire 5–50 °C range.

Difference UV and Circular Dichroism Spectra. In Figure 4 are shown the difference ultraviolet spectra between the free enzyme and the carbamoyl phosphate bound enzyme for both the wild type and the mutant. The UV absorption spectrum of the wild-type enzyme undergoes pronounced changes in the 250–310-nm range when carbamoyl phosphate binds. Distinct fine structure is seen with sharp maxima at 272, 281, 287, and 294 nm, superimposed on a broader but characteristic absorbance change that extends from 250 to 320 nm. These features are reproducible with different preparations of enzyme, and the spectral features remain nearly constant from pH 6.5 to pH 9. The intensity of the fine structure increases slightly when the level of carbamoyl phosphate was raised from 0.1 to 5 mM, which is consistent with a K_{ia} of 0.046 mM for this compound. Difference spectra are also obtained for the wild-type enzyme in the presence of inorganic phosphate, indicating that this compound also induces conformational changes in the enzyme (A. W. Miller and L. Kuo, unpublished data). When carbamoyl phosphate is present, addition of L-norvaline results in a slight change in the height of the fine structure peaks and shifts of up to 0.5 nm in their positions. However, in the absence of carbamoyl phosphate, addition of up to 1 mM L-ornithine, or L-norvaline, to the wild-type solution yields a flat difference spectrum. In striking contrast, absorbance changes and spectral fine structure are completely absent for the Gly-57 mutant in the presence of carbamoyl phosphate or inorganic phosphate irrespective of the presence of L-norvaline. The absence of difference UV suggests that carbamoyl phosphate (and inorganic phosphate) no longer induces conformational changes in the mutant.

Finally, we have also recorded the circular dichroic spectra in the 205–250-nm range of the wild type and the Gly-57 mutant to determine if there are disruptions of the native conformation of ornithine transcarbamoylase arising from the point mutation. The spectra of molar ellipticity of the two enzymes are essentially identical in shape and amplitude except in the 210–212-nm and 228–232-nm regions where a 6–7% difference in molar ellipticity is seen. This difference is within the error of the experiment (figure not shown).

Discussion

To attribute alterations of kinetic parameters to mutation of a specific enzymic residue, it is first necessary to demonstrate that (1) there is only a single designated mutation and (2) the purified mutant enzyme preparation does not contain any wild-type protein. The second consideration is particularly important for mutant enzymes that are minimally active. The G1pUC18m2 mutant plasmid was transformed into TB2 cells from which the mutant ornithine transcarbamoylase was obtained (see Materials and Methods). To ensure that only a single base substitution of C → G was made at nucleotide position 169 of *argI*, we isolated G1pUC18m2 from the same colony of TB2 from which the mutant enzyme was purified, extracted *argI*, and recloned it into M13mp19 for DNA sequencing of the entire *argI* gene. We found unequivocally only the intended C → G change at nucleotide position 169; thus, our mutant TB2 culture does not contain both mutant and wild-type *argI* plasmid. Furthermore, the dissociation constant of carbamoyl phosphate and the inhibition constants and patterns of the mutant are different from those for the wild type (Tables II and III). Since these parameters are characteristic of an enzyme rather than its concentration, they would not have differed if the activity observed for the mutant preparation were due to a mixture of inactive mutant and active wild-type enzymes.

Change in k_{cat}. The substitution of glycine for arginine at residue 57 of ornithine transcarbamoylase results in a drop of turnover number from 1.4×10^5 min^{-1} to 6.6 min^{-1}. Two obvious possibilities, a change in pH optimum or a loss of conformational integrity, that might have caused the severe loss of activity can be ruled out immediately.

The low catalytic efficiency found for the mutant is not due to measurement of its activity at a pH where the enzyme is inactive. Kinetic assays conducted on the mutant enzyme between pH 5.5 and pH 10.5 show that the pH range for maximal activity is between 7.5 and 9.0, similar to that of the wild type (Kuo et al., 1985).

The fact that the K_M^{app} values of the substrates are little or not at all changed for the mutant (Table I and Figure 3), that the circular dichroisms of the mutant and the wild type are identical, and that the mutant has the same intrinsic extinction coefficient at 280 nm as wild type all support the conclusion that the mutant enzyme retains the same overall three-dimensional structure of the wild type. The secondary structures and their gross spatial arrangement in the mutant have apparently not been unduly perturbed. However, this conclusion must not be overstated. It is possible that small, or spectroscopically silent, but kinetically important structural changes within the active site exist due to the complete removal of the arginine side chain. Local structural perturbations in the active site cannot be assessed without X-ray diffraction data, but the K_M^{app} values of both substrates indicate that insofar as the enzyme being saturated by the substrates for turnover is concerned there is little change between the two enzymes.

Change in Kinetic Mechanism. The inhibition patterns described under Results identify the kinetic mechanism of the mutant as rapid-equilibrium random Bi Bi in contrast to the steady-state ordered Bi Bi mechanism for the wild type. Our treatment of the random mechanism as a rapid-equilibrium one is further justified by the loss of substrate inhibition (Cleland, 1979) by ornithine, the absence of breaks in the plots of log K_M^{app} versus $1/T$, and the extremely slow turnover rate.

To our knowledge, an alteration of substrate binding order resulting from substitution of a single enzymic residue has not been reported previously.

We note that experiments with the Gly-57 mutant in the pH 7–10 range show no difference from the kinetic patterns observed at pH 8.5 (I. Zambidis and L. Kuo, unpublished results).

The breakdown of the obligate binding order implies that the wild-type enzyme is inherently capable of binding ornithine as the first substrate. This property is not uncommon, and many enzymes that observe ordered mechanism display some random binding of substrates. Stalon and co-workers have concluded that ornithine transcarbamoylase from *E. coli* W observes a Theorell–Chance ordered binding mechanism at pH 8 but not at pH 6.8. At the lower pH, their data reveal considerable random binding although carbamoyl phosphate is still the preferred first substrate (Wargnies et al., 1978). Likewise, for the catalytic subunit of aspartate transcarbamoylase, which is usually believed to follow an ordered mechanism, Morrison and co-workers have found random binding under some conditions, especially for the poor substrate acetyl phosphate whose mechanism is treated as a rapid-equilibrium one (Heyde et al., 1973; Heyde & Morrison, 1973).

Two possibilities may account for the transformation of substrate binding order. First, while ornithine binds appreciably to *only* the binary ES complex in the wild-type reaction, it now binds comparably to *both* the free enzyme and the binary complex in the mutant reaction. In other words, there is a drastic loss in ornithine affinity of the ES complex relative to that of the free enzyme as a result of the mutation. Another view is that there is a decreased affinity of carbamoyl phosphate relative to that of ornithine as first substrate. These interpretations are supported by the change in affinity of the substrates to the two enzymes (Table III) and by the difference UV spectra (Figure 4). A less likely alternative is that the rate-determining step of the enzymic reaction is altered by the mutation and chemical transformation of the ternary complex or the release of the first product becomes rate limiting. Although the rate-limiting step is not known for either enzyme at present, the data shown in Figure 2 strongly suggest that it is the same for the two enzymes: (1) the log k_{cat} versus $1/T$ plots are linear for both enzymes over an extended temperature range indicating one rate constant dictates k_{cat};[4] (2) the corresponding ΔH_{act} values observed for k_{cat} are 11 and 10 kcal/mol, suggesting that the same rate-limiting step governs both the wild-type and the mutant reactions. It is unlikely that the rate-limiting step has been altered while the ΔH_{act} remains the same.

Change in Binding Constants. To compare the affinities of the two enzymes toward substrates, we employ the binding constants measured from steady-state kinetic assays. We stress that the use of K_M^{app} as an indicator of substrate affinity is an erroneous common practice. While it is well-known that only in rare cases, for example, an enzyme observing the simple Michaelis–Menten mechanism, can this parameter be a valid gauge of substrate affinity, the ease of K_M^{app} determination renders its use an irresistible exercise. The complication of using Michaelis constants as a measure of enzyme specificity has been discussed as early as 1964 by Bender, Kezdy, and Gunter (Bender et al., 1964). Nevertheless, the dissociation constants of substrates are often difficult to measure, and the binding strength of a substrate in *equilibrium* with an enzyme is not necessarily relevant to activity because thermodynamic equilibrium is often not attained in an enzyme reaction. For these reasons and when direct estimates of binding constants from kinetic assays are not possible, the use of substrate analogues under steady-state turnover conditions provides a suitable means to evaluate the stereospecificity and affinity of a substrate to an enzyme.

The binding constants for the mutant and wild-type enzymes are given in Table III. The comparisons of most interest are the binding of carbamoyl phosphate to the free enzyme and of ornithine to the enzyme–carbamoyl phosphate complex. The affinity of carbamoyl phosphate to the free enzyme is 70 times weaker for the mutant (K_{ia} = 3.2 mM) than for the wild type (K_{ia} = 0.046 mM), suggesting that Arg-57 is salt linked to the phosphate moiety of the substrate. Since inorganic phosphate also binds weaker to the mutant with a 40-fold increase in inhibition constant (K_i) from 0.13 to 5 mM, *Arg-57 is important in stabilizing the phosphate moiety in both the initial and final stages of transcarbamoylation.* It is difficult to assess without structural information and kinetic data of other point mutants at the phosphate site[1] why this mutation has a greater effect on carbamoyl phosphate than on inorganic phosphate. Arg-57 is not the only positively charged residue at the phosphate site, and the binding geometry of the phosphate moiety of carbamoyl phosphate may not be identical with that of inorganic phosphate. We are in the process of synthesizing the transition-state analogue *N*-(phosphonacetyl)-L-ornithine, to test for possible interactions between Arg-57 and the phosphate group in the transition state of the reaction. Such interactions may contribute to the chemical transformation of substrates to products.

The strength of L-ornithine binding to the enzyme-carbamoyl phosphate complex is given for the mutant by K_b = 0.17 mM, but K_b cannot be assessed for the wild type for which the rapid-equilibrium assumption does not obtain. One way to estimate ornithine affinity is to compare the binding of L-norvaline to the two enzymes; this is justified because norvaline is a classical competitive inhibitor (Fromm, 1979) of ornithine. Norvaline is structurally identical with ornithine except that it lacks the δ-amino group (the part of the molecule that actually reacts). We have shown previously that L-ornithine binds the binary ES complex of the wild type productively only in zwitterionic form with an uncharged δ-amino group (Kuo et al., 1985). Thus, there is no ionic contribution from the δ-amino of ornithine to binding, at least in the case of the wild type.

Norvaline binds the wild-type binary complex (K_{ii} = 54 μM) about 500 times stronger than its binding to the binary complex of the mutant (K_{ii} = 25 mM). This major change in affinity is surprising since Arg-57 is not expected to be proximal to the ornithine site. Our result suggests that *the ornithine site in the binary enzyme–carbamoyl phosphate complex is indirectly altered by the mutation.* For the wild type, ornithine and norvaline can only bind after carbamoyl phosphate. The ordered binding pattern implies that carbamoyl phosphate induces requisite conformational changes of the active site and/or upon binding forms part of the ornithine site. A characteristic fine structure, arising from perturbations of tyrosyl and tryptophanyl residues upon carbamoyl phosphate binding, is observed in the difference UV spectrum of the wild type (Figure 4) revealing conformational changes in the active site induced by this substrate. The same "induced-fit" tran-

[4] In the unlikely event that two or more microscopic steps are rate controlling, their temperature dependence must be sufficiently the same within the temperature range examined so that the Arrhenius relationship is observed for k_{cat}. This situation does not alter the interpretation presented.

sition is apparently not reached in the mutant. Carbamoyl phosphate binds the mutant 70 times weaker, and no difference spectrum is observed when this substrate binds the mutant. As a result, in the mutant reaction ornithine now binds poorly to not only the free enzyme (2.3 mM) but also the binary complex (0.17 mM) giving rise to a random addition mechanism.

Using the dissociation constants of carbamoyl phosphate and L-norvaline, the loss in apparent binding energy due to the mutation may be calculated. With 1 M as the standard state, alteration of Arg-57 to glycine leads to a loss of 2.5 kcal/mol in carbamoyl phosphate binding and 3.6 kcal/mol in L-norvaline binding. *If* L-ornithine binding is similarly affected by the mutation, a similar drop in binding energy is expected, and the dissociation constant of ornithine from the ternary complex would be 0.37 μM. This affinity is consistent with substrate affinity found in other Bi Bi enzyme systems, e.g., tyrosyl-tRNA synthetase (Wells & Fersht, 1986).

Change in Entropy. The difference in catalytic efficiency between the two enzymes is entropic in origin. With use of the turnover numbers of the two enzymes and the ΔH_{act} values obtained from either ornithine or carbamoyl phosphate saturation studies, the calculated ΔS_{act} for the wild-type reaction is −5.5 eu while that for the mutant is −29 eu (Table IV). *This change in ΔS_{act} accounts for a loss of 6–7 kcal/mol in the activation of the rate-determining step* and is the source of the drop in the turnover number.

Proper juxtaposition of reaction centers of substrates is an important aspect of enzymic catalysis. Obviously, substrates must bind sufficiently tightly so as not to diffuse away from the active site before chemical transformation. The binding energy observed in equilibrium and/or kinetic assays is an apparent one, so it is difficult to assess from binding or kinetic studies how much of the intrinsic binding energy of a substrate is utilized in promoting tight binding of the transition state.[5] We have shown that only the zwitterionic species of L-ornithine with a lone pair of electrons on the δ-amino group binds *productively* to the active site (Kuo et al., 1985). Charge polarization of the carbonyl of carbamoyl phosphate may be essential to further assist nucleophilic attack by the δ-amino of ornithine. The bond-breaking process of phosphate from carbamoyl phosphate should not be rate affecting as the ester linkage is a high-energy bond, although the departing PO_4^{3-} needs to abstract a proton which may be donated by a general acid enzymic group or a proton on the δ-nitrogen atom of ornithine in the tetrahedral intermediate. In other words, with a strong nucleophile and a good leaving group, structural distortions of substrates do not appear to be important for transcarbamoylation, and transformation of the ternary complex from substrate to product is unlikely to be rate determining. Conformational transitions leading to proper stereospecific binding of L-ornithine or conformational changes associated with release of inorganic phosphate may be much more important in the catalysis of ornithine transcarbamoylase.

Conclusions

On the basis of these thermodynamic, spectroscopic, and kinetic data, we conclude that without Arg-57 the enzyme does not bind carbamoyl phosphate as tightly. Further, this enzyme cannot undergo the conformational transitions necessary to promote productive binding of L-ornithine for efficient catalysis. This encumbrance leads to a loss of 6–7 kcal/mol in the transition state associated with the rate-determining step of the reaction, and the normally ordered substrate binding to the enzyme becomes random. Without assessments of other essential active site residues and in the absence of high-resolution crystallographic structure, it is difficult to pinpoint further the sequence of events responsible for affinity and specificity. Our present single crystals of ornithine transcarbamoylase diffract to better than 3-Å resolution. We have also obtained small, but presently unusable, single crystals of the Gly-57 mutant. The work presented here represents our first step in elucidating the mechanism of action of ornithine transcarbamoylase with the aid of site-directed mutagenesis.

[5] See Fersht (1988) for a discussion on the relationships between apparent binding energies measured in site-directed mutagenesis experiments and the energetics of binding and catalysis.

Acknowledgments

We thank Professor James Wild of Texas A&M University for his gift of pAI101, Professor Evan R. Kantrowitz of Boston College for helpful discussion on mutagenesis techniques, Dr. Mary T. Walsh of Boston University School of Medicine for help in circular dichroic measurements, and Connie Caron for technical assistance in DNA sequencing.

Registry No. EC 2.1.3.3, 9001-69-8; L-Arg, 74-79-3; Arg-57 WT, 117183-18-3; Gly-57 mutant, 117183-19-4; $H_2NCO_2PO_3H_2$, 590-55-6; L-ornithine, 70-26-8; inorganic phosphate, 14265-44-2; L-norvaline, 6600-40-4.

References

Bencini, D. A., Houghton, J. E., Hoover, T. A., Foltermann, K. F., Wild, J. R., & O'Donovan, G. A. (1983) *Nucleic Acids Res. 11*, 8509–8518.

Bender, M. L., Kezdy, F. J., & Gunter, C. R. (1964) *J. Am. Chem. Soc. 86*, 3714–3721.

Carter, P., Bedouelle, H., & Winter, G. (1985) *Nucleic Acids Res. 13*, 4431–4443.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 500–513.

Davis, L. G., Dibner, M. D., & Battey, J. F. (1986) in *Methods in Molecular Biology*, pp 91–92, 253–255, Elsevier Science, New York.

Fersht, A. R. (1988) *Biochemistry 27*, 1577–1580.

Fortin, A. F., Hauber, J. M., & Kantrowitz, E. R. (1981) *Biochim. Biophys. Acta 662*, 8–14.

Fromm, H. J. (1979) *Methods Enzymol. 63*, 467–486.

Heyde, E., & Morrison, J. F. (1973) *Biochemistry 12*, 4727–4732.

Heyde, E., Nagabhushanam, A., & Morrison, J. F. (1973) *Biochemistry 12*, 4718–4726.

Holland, P. H., & Welsch, R. E. (1977) *Commun. Statistics: Theory Methods 6*, 813–827.

Horwich, A. L., Fenton, W. A., Williams, K. R., Kalousek, F., Kraus, J. P., Doolittle, R. F., Konigsberg, W., & Rosenberg, L. E. (1984) *Science (Washington, D.C.) 224*, 1068–1074.

Houghton, J. E. (1986) Ph.D. Dissertation, Texas A&M University.

Houghton, J. E., Bencini, D. A., O'Donovan, G. A., & Wild, J. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4864–4868.

Krause, K. L., Volz, K. W., & Lipscomb, W. N. (1987) *J. Mol. Biol. 193*, 527–553.

Kuo, L. C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5243–5247.

Kuo, L. C., Lipscomb, W. N., & Kantrowitz, E. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2250–2254.

Kuo, L. C., Herzberg, W., & Lipscomb, W. N. (1985) *Biochemistry 24*, 4754–4761.

Legrain, C., & Stalon, V. (1976) *Eur. J. Biochem. 63*, 289–301.

Legrain, C., Halleux, P., Stalon, V., & Glansdorff, N. (1972) *Eur. J. Biochem. 27*, 93-102.
Maniatus, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Handbook*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Marshall, M., & Cohen, P. P. (1972) *J. Biol. Chem. 247*, 1654-1668.
Marshall, M., & Cohen, P. P. (1977) *J. Biol. Chem. 252*, 4276-4286.
Marshall, M., & Cohen, P. P. (1980a) *J. Biol. Chem. 255*, 7296-7300.
Marshall, M., & Cohen, P. P. (1980b) *J. Biol. Chem. 255*, 7301-7305.
Nakamura, M., & Jones, M. E. (1970) *Methods Enzymol. 17A*, 286-294.
Norris, K., Norris, F., Christiansen, L., & Fiil, N. (1983) *Nucleic Acids Res. 11*, 5103-5112.
Pastra-Landis, S. C., Foote, J., & Kantrowitz, E. R. (1981) *Anal. Biochem. 118*, 358-363.
Porter, R. W., Modebe, M. O., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1846-1859.
Sachsenheimer, W., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 23-36.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Schachman, H. K., Pauza, C. D., Navre, M., Karels, M. J., Wu, L., & Yang, Y. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 115-119.
Schleif, R. F., & Wensink, P. C. (1984) in *Practical Methods in Molecular Biology*, p 196, Springer-Verlag, New York.
Schulz, G. E., & Schirmer, R. H. (1979) in *Principles of Protein Structure*, p 226, Springer, New York.
Vogelstein, B., & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 615-619.
Volz, K. W., Krause, K. L., & Lipscomb, W. N. (1986) *Biochem. Biophys. Res. Commun. 136*, 822-826.
Wargnies, B., Legrain, C., & Stalon, V. (1978) *Eur. J. Biochem. 89*, 203-212.
Wells, T. N. C., & Fersht, A. R. (1986) *Biochemistry 25*, 1881-1886.
Zoller, M. J., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487-6500.

Specific Inhibition of DNA Biosynthesis Induced by 3′-Amino-2′,3′-dideoxycytidine†

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Received March 30, 1988; Revised Manuscript Received July 29, 1988

ABSTRACT: 3′-Amino-2′,3′-dideoxycytidine (3′-NH_2-dCyd) produced an S-phase-specific block in exponentially growing L1210 leukemia cells. The monophosphate and triphosphate forms of the drug were detected within a few hours of 3′-NH_2-dCyd treatment of intact cells. No significant change in the deoxynucleoside triphosphate levels was observed during the early stages of treatment. However, by 24 h a 2-fold increase in the amount of the deoxynucleoside triphosphates was seen. The triphosphate form of the drug competitively inhibited dCTP incorporation into calf thymus DNA using highly purified DNA polymerase α. The K_i was determined to be 9.6 μM with respect to dCTP. Incorporation of the analogue into DNA was not detected. On the other hand, sucrose gradient analysis suggested that incorporation of the analogue into actively synthesized DNA may account for the biological activity of this compound. Treatment with 3′-NH_2-dCyd induced single-strand breaks in actively synthesized DNA, but no double-strand breaks were observed in the presence of the analogue. The data indicate that 3′-amino-2′,3′-dideoxycytidine specifically interferes with DNA replication at the level of DNA polymerase by inhibiting chain elongation.

ara-C[1] is the prototype of deoxycytidine analogues used in the treatment of neoplastic disease. This agent is S-phase specific (Chu & Fischer, 1962), and the active metabolite, *ara*-CTP, exerts its action by inhibition of DNA biosynthesis (Momparler, 1969; Furth & Cohen, 1968; Chu & Fischer, 1968; Durham & Ives, 1969). *ara*-C has been shown by use of intact cells to be readily incorporated into DNA by a number of investigators (Graham & Whitmore, 1970; Manteuil et al., 1974; Kufe et al., 1980). Recently, the level of *ara*-CMP incorporation into DNA was correlated with the loss of clonogenic survival of both L1210 murine leukemic cells (Kufe et al., 1980) and HL-60 human promyelocytic leukemic

†This investigation was supported by U.S. Public Health Service Grants CA-40283, CA 05262, and CA 45410 from the National Cancer Institute. M.S.W. is a recipient of an Advanced Predoctoral Fellowship in Pharmacology/Toxicology from the Pharmaceutical Manufacturers Association Foundation.

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[1] Abbreviations: *ara*-C, 1-β-D-arabinofuranosylcytosine; 3′-NH_2-dCyd, 3′-amino-2′,3′-dideoxycytidine; ddCyd, 2′,3′-dideoxycytidine; *ara*-CMP, 1-β-D-arabinofuranosylcytosine 5′-monophosphate; 3′-NH_2-dCMP, 3′-amino-2′,3′-dideoxycytidine 5′-monophosphate; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; dNTP, deoxynucleoside 5′-triphosphate; 5-aza-dCyd, 5-aza-2′-deoxycytidine; 3′-NH_2-dThd, 3′-amino-3′-deoxythymidine. For the triphosphates of the analogues, the M in the abbreviation is replaced by T.

0006-2960/88/0427-8832$01.50/0