Chemical Rescue by Guanidine Derivatives of an Arginine-Substituted Site-Directed Mutant of *Escherichia coli* Ornithine Transcarbamylase[†]

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ABSTRACT: *Escherichia coli* ornithine transcarbamylase (OTCase) catalyzes the production of L-citrulline and phosphate from carbamyl phosphate and L-ornithine in L-arginine biosynthesis. We show that exogenous guanidines can restore activity to (chemically rescue) a catalytically-impaired site-directed mutant OTCase, R57G, in which glycine replaces an an active site arginine. The best rescue agent is guanidine hydrochloride, which enhances the rate of the mutant 2000-fold. The turnover number for the guanidine-rescued R57G mutant is 10% that of wild-type. The addition of guanidine to the R57G mutant has little effect on K_M^{CP} values, and the rescue effect is therefore attributed principally to an increase in k_{cat}. Other compounds were screened as potential rescue agents, but rate enhancement is highly selective for guanidines. Not all guanidines show large increases in k_{cat}. For a comparative series that includes guanidine and alkylguanidines, substituent size is inversely related to k_{cat}. Brønsted analysis of guanidines with varying pK_a values indicates that a partial positive charge is implicated in rescue, consistent with the proposed role of arginine 57 in catalysis. In UV difference and ^{31}P-NMR spectra, carbamyl phosphate-induced effects associated with wild-type OTCase are observed in the R57G mutant only in the presence of guanidine. The kinetic mechanism of the mutant is random in the presence or absence of guanidine, in contrast to the sequential ordered mechanism of the wild-type enzyme. Thus, chemical rescue of R57G by guanidine hydrochloride restores many but not all wild-type properties to the mutant enzyme.

Ornithine transcarbamylases (OTCases; ornithine carbamoyltransferase; EC 2.1.3.3) constitute a family of metabolic enzymes that are found in both prokaryotes and eukaryotes. *E. coli* OTCase, the subject of this study, catalyzes a step in the biosynthesis of arginine. The corresponding enzyme from ureotelic animals is located in the mitochondrial matrix and participates in the urea cycle. Both of these enzymes are obligately anabolic. Some bacteria, such as *P. aeruginosa*, possess both catabolic and anabolic OTCases that are regulated through different physiological mechanisms. OTCases exhibit considerable sequence homology, and residues with putative catalytic roles are conserved. Anabolic OTCases, including the *E. coli* and mammalian enzymes, are active as homotrimers with subunit molecular masses near 37 kDa, while catabolic OTCases may form higher-order oligomers. OTCase exhibits approximately 40% identity with the catalytic subunit of aspartate transcarbamylase, ATCase (Horwich et al., 1984). ATCase catalyzes an analogous reaction to that of OTCase in which carbamyl phosphate and L-aspartate, rather than L-ornithine, are substrates. It has been suggested that OTCase and ATCase share a common evolutionary origin (Houghton et al., 1984).

E. coli OTCase has been well-characterized with respect to its enzymatic properties and provides a good mechanistic model for other anabolic OTCases. The enzyme catalyzes the following reaction in the forward direction:

carbamyl phosphate + L-ornithine ⇌ L-citrulline + inorganic phosphate

E. coli OTCase displays an extremely high substrate specificity (Kuo et al., 1985) and exhibits Michaelis−Menten kinetics. Substrate binding is sequential, with carbamyl phosphate binding before L-ornithine (Marshall & Cohen, 1972; Legrain & Stalon, 1976; Wargnies et al., 1978). Binding of carbamyl phosphate, in the neutral pH range, causes the enzyme to undergo a conformational change that can be observed through ultraviolet (UV)[1] difference spectroscopy (Miller & Kuo, 1990). Kinetic findings suggest that the carbamyl phosphate-induced conformational transition of OTCase may be essential to achieve a high rate of substrate turnover (Zambidis & Kuo, 1990). It has been found that arginine 57, a conserved residue found in all OTCases, is essential to this conformational change. When arginine 57 is replaced by a glycine (R57G) or by a histidine (R57H) through site-directed mutagenesis, the substrate-induced conformational change is absent. Relative to wild-type, the k_{cat} is lowered 4 orders of magnitude, from 1.3×10^5 min^{-1} to 7 or 47 min^{-1}, for R57G or R57H, respectively; and K_M^{CP} increases from 0.05 mM to 0.26 or 0.75 mM,

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[1] Abbreviations: UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NMR, nuclear magnetic resonance.

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respectively (Kuo et al., 1988; Goldsmith & Kuo, 1993).[2] Ornithine binding becomes nonstereospecific, and both the anionic and zwitterionic species can bind the enzyme, whereas the wild-type enzyme prefers the zwitterionic species (Goldsmith et al., 1991).

To further investigate the catalytic mechanism of *E. coli* OTCase, we have used chemical rescue of the R57G site-directed mutant. The term "chemical rescue" refers to the introduction of exogenous ligands to inactive mutant enzymes to restore catalytic activity. Toney and Kirsch (1989) pioneered this approach as a tool for the systematic study of enzyme catalysis. The expectation that exogenous agents can mimic and compensate for the lost functionality of the substituted side chain appears to be borne out experimentally. The majority of chemical rescue studies have used amines or imidazoles to rescue various enzyme mutants with substituted lysine (Toney & Kirsch, 1989; Smith & Hartman, 1991; Zhukovsky et al., 1991; Sekimoto et al., 1993; Harpel & Hartman, 1994) or histidine (Tu & Silverman, 1989; den Blaauwen et al., 1991; Carter et al., 1991) residues, respectively. Phillips et al. (1992) showed that guanidine, methylguanidine, or ethylguanidine restored activity to an arginine-substituted carboxypeptidase mutant. Phenols or acetate have been employed to rescue mutant enzymes with a substituted tyrosine (Brooks & Benisek, 1992) or aspartic acid (Perona et al., 1994) residue. In each of these cases, the chemical rescue agent contains the same functional group as that of the substituted amino acid residue.

Toney and Kirsch (1989) showed that a classical Brønsted analysis could be used to probe transition-state structure in enzyme-catalyzed reactions. This type of linear free energy analysis of structure−reactivity correlations has long been utilized in physical organic chemistry. Through the systematic variation of catalysts and determination of rate constants, the Brønsted analysis provides a quantitative measure of the role played by charge in the transition state of a chemical reaction. The inherent limitation in using the Brønsted analysis to study enzyme reaction mechanisms is that the enzyme itself provides the catalytic species, which cannot be varied during the experiment. However, Toney and Kirsch showed that in the case of chemical rescue, site-directed mutant enzymes can be studied in such a manner with exogenous ligands serving as the catalysts to be varied. To date, relatively few chemical rescue studies (Ehrig et al., 1991; Sekimoto et al., 1993; Harpel & Hartman, 1994) have included a Brønsted analysis. In the present studies, we have investigated a range of guanidines as rescue agents for the the R57G OTCase mutant. Structure−activity analysis of these data shows the contributions of size and pK_a to the rescue phenomenon. We propose that in this mutant the rescue agent binds at the site occupied by the arginine side chain in the wild-type enzyme.

MATERIALS AND METHODS

Carbamyl phosphate, L-ornithine, hydroxyapatite, and n-butylamine were purchased from Sigma Chemical Co. and used without further purification except as noted. Ethanolamine was purchased from Eastman Kodak Co. Methylguanidine hydrochloride, ethylguanidine sulfate, aminoguanidine hydrochloride, nitroguanidine, propylamine, cyanamide, *N*-guanylurea sulfate hydrate, 1,3-diaminoguanidine monohydrochloride, and *O*-methylisourea hydrogen sulfate were purchased from Aldrich Chemical Co. The R57G cell line was a gift from Dr. L. C. Kuo.

Cell Growth and Protein Purification. The R57G mutant is overexpressed in TB2 cells in plasmid G1pUC18m2 (Kuo et al., 1988). The cells were grown overnight in LB medium containing 100 mg/L ampicillin at 37 °C. The cells were harvested by centrifugation, sonicated, and centrifuged again. The supernatant was heated at 65 °C for 10 min and recentrifuged. Ammonium sulfate was added to the supernatant to 40% saturation and centrifuged. The supernatant was then brought to 90% saturation at 4 °C. The pellet was resuspended in a small volume of water and diluted by the addition of 2 volumes of buffer (20 mM Tris-HCl, pH 8.0, 0.5 M ammonium sulfate). This was loaded onto a phenyl-sepharose (Pharmacia) column equilibrated with the same buffer. The OTCase was eluted by a gradient to 20 mM Tris-HCl, pH 8.0, 25 mM ammonium sulfate. The OTCase-containing fractions were dialyzed against a buffer of 20 mM Tris-HCl, pH 8.0, 75 mM NaCl. This was loaded onto a DE-52 (Whatman) column equilibrated in the same buffer, and eluted with a gradient to 20 mM Tris-HCl, pH 8.0, 300 mM NaCl. The active fractions were pooled and dialyzed into 25 mM sodium phosphate, pH 6.3, buffer. The pool was mixed with approximately 15 mL of wet hydroxyapatite, stirred for 15 min, and filtered. The OTCase was batch-eluted by addition of 50 mM sodium phosphate buffer, pH 6.3, to the damp hydroxyapatite. The eluant was concentrated to 30 mg/mL and exchanged into a buffer of 20 mM Tris-HCl, pH 8.0, 0.1% NaN_3, and 1 mM EDTA. The purified OTCase stains as a single band on Coomassie-stained SDS−PAGE gels.

Synthesis of Substituted Guanidines. Guanidine compounds that were not commercially available were synthesized from *O*-methylisourea hydrogen sulfate and the corresponding amine. The *O*-methylisourea hydrogen sulfate was converted to *O*-methylisourea sulfate by reaction with cyanamide and methanol as described (Weiss & Krommer, 1974). Propylguanidine (mp 229−232 °C), butylguanidine (mp 206−208 °C), and guanidinoethanol (mp 114−117 °C) were prepared as sulfate salts as outlined in Weiss and Krommer (1974). Elemental analysis was performed by Galbraith Laboratories, Inc. (Knoxville, TN). For propylguanidine ($C_8H_{24}N_6SO_4$): calculated, C, 31.99; H, 8.05; N, 27.98; S, 10.67; observed, C, 32.59; H, 8.25; N, 27.13; S, 10.45. For butylguanidine ($C_{10}H_{28}N_6SO_4$): calculated, C, 36.57; H, 8.58; N, 25.59; S, 9.76; observed, C, 36.35; H, 8.61; N, 24.84; S, 9.76. For guanidinoethanol ($C_6H_{20}N_6SO_4$): calculated, C, 23.68; H, 6.62; N, 27.62; S, 10.54; observed, C, 23.71; H, 6.80; N, 26.79; S, 10.62.

Enzyme Assays. OTCase activity was measured by phosphate production as determined by the method of Bencini et al. (1983). Recrystallization of carbamyl phosphate from 50% ethanol at −20 °C did not significantly decrease the background of this assay, so carbamyl phosphate was used without further purification. The assays were run in 50 mM Tris−acetate, pH 8.3, in a water bath kept constant at 25 °C. In assays of various rescue agents, the carbamyl phosphate concentration was 20 mM, and the ornithine concentration was 4 mM, as these concentrations were determined beforehand to be saturating levels. The concen-

[2] These values are from the literature cited. The corresponding values we measured for the R57G mutant are $k_{cat} = 5.7$ min^{-1} and $K_M^{CP} = 0.22$ mM, in good agreement with literature values. Unless otherwise noted, values of kinetic data throughout this paper derive from our measurements.

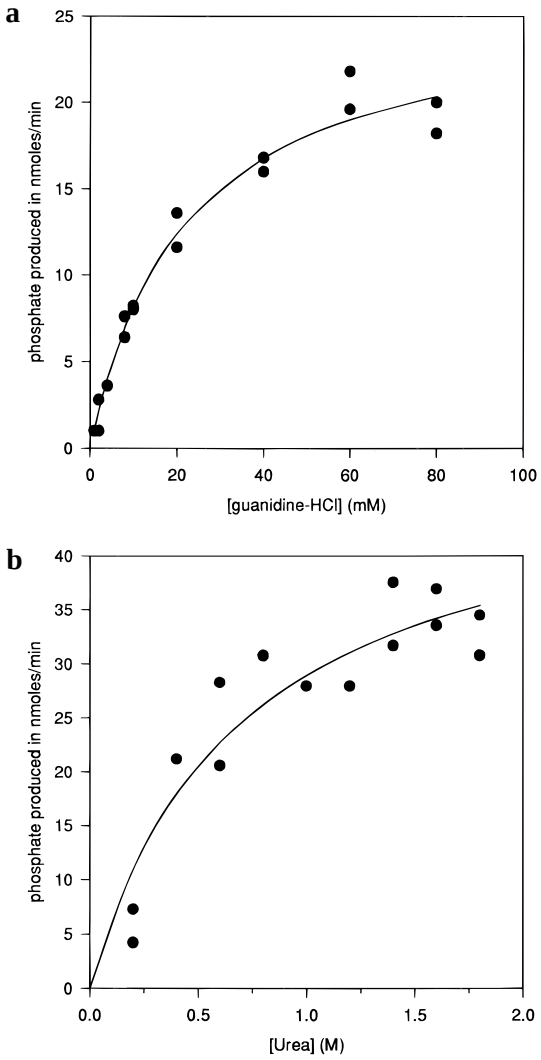

FIGURE 1: Determination of K_R for OTCase R57G mutant rescue for (a) guanidine hydrochloride and (b) urea. Assay conditions were as described under Materials and Methods. OTCase concentrations were 480 ng/mL or 750 μg/mL for guanidine hydrochloride or urea, respectively. Reaction rates were calculated in nanomoles of phosphate produced per minute after subtraction of background. For urea, initial rates were determined from time points $\leq$30 s to avoid errors due to denaturation at higher urea concentrations. Reaction rates were linear in this time range.

tration of the rescue agent varied in carbamyl phosphate assays from 40 mM to as high as 1.8 M, representing the concentration that effected that maximal rate enhancement for each specific rescue agent. Initial rate data were collected by starting the reaction by addition of carbamyl phosphate, then removing samples and quenching in cold color reagent (100 mM $ZnCl_2$, 15 mM ammonium molybdate, pH 5.0, at 4 °C), and reading the absorbance at 360 nm 1 min after quenching. k_{cat} and K_M^{CP} were determined by Lineweaver−Burk analysis. Values for K_R, defined as the concentration of rescue agent that gives half-maximal rate enhancement, were determined by fitting the data from the assays of the rescue agents to the equation: $v = V$[rescue agent]/(K_R + [rescue agent]), following the approach of Harpel and Hartman (1994). Saturation curves for guanidine hydrochloride and urea are shown in Figure 1.

Statistical Analysis. The values of all parameters used in this study appear in Table 1. The pK_a values are from Perrin (1965). For ethylguanidine, propylguanidine, butylguanidine, and guanidinoethanol, pK_a was determined through potentiometric titration of the guanidine with sodium hydroxide.

Table 1: pK_a Values and Steric Parameters Used for Analyses[a]

compound	pK_a	E_S
guanidine	13.6	0
methylguanidine	13.4	−1.24
ethylguanidine	13.3	−1.31
propylguanidine	13.5	−1.60
butylguanidine	13.2	−1.62
guanidinoethanol	13.1	−2.20
nitroguanidine	12.2	−2.52
aminoguanidine	11.0	−0.61
guanylurea	3.9	−0.88
urea	0.2	0

[a] The values are from Hansch and Leo (1979) and Perrin (1965) except where noted under Materials and Methods.

The values of the Taft steric parameter, E_S, are from Hansch and Leo (1979). For guanylurea and guanidinoethanol, the Taft steric parameter was estimated by taking the van der Waals radius (Bondi, 1964) of the first atom of the substituent (Hansch & Leo, 1979) and applying the equation of Kutter and Hansch (1969). The same value for E_S was used for both guanidine and urea, since the guanidino amino group and the carbonyl oxygen of urea are equivalent for this analysis. The Taft steric parameter for the disubstituted 1,3-diaminoguanidine could not be determined straightforwardly as for the monosubstituted compounds and was omitted from the statistical analysis.

For the statistical analyses, K_R values were calculated as the free base concentrations at pH 8.3. The k_{cat} values used were calculated as k_{cat}(obs) − k_{cat}(no rescue). Kinetic data were plotted against E_S or pK_a, and the slopes α and β were determined by linear regression on the equation: $\log k_{cat}/K_R = \alpha(E_S) + \beta(pK_a) + C$, using E_S and pK_a as independent variables (Toney & Kirsch, 1989).

UV Difference Spectra. Spectra were taken as described previously (Miller & Kuo, 1990) except that in this study 60 mM guanidine hydrochloride was added to the R57G sample. Spectra were recorded on a Perkin-Elmer Lambda 2 UV−visible spectrophotometer with a scan speed of 15 nm/min.

RESULTS

The effect of guanidine hydrochloride on R57G OTCase reaction rates can be seen in the titration plot (Figure 1a). When carbamyl phosphate and ornithine are saturating, increasing the amount of guanidine added to the solution greatly enhances the rate of the reaction. No rate enhancement is observed if wild-type OTCase is substituted for the R57G mutant. The pH of the solution is unchanged by the addition of guanidine hydrochloride up to 1 M concentration. The observed rate enhancement is highly selective for guanidines, as no significant rate enhancement was observed using propylamine, pyridine, imidazole, ammonium acetate, ethanolamine, or L-arginine. For guanidine hydrochloride, K_R was found to be 22 ± 3 mM.

The reaction rate was measured in the presence of saturating levels of guanidine and ornithine, with varying subsaturating levels of carbamyl phosphate. Data were then plotted in a Lineweaver−Burk plot to obtain the kinetic constants K_M^{CP}, V_{max}, and k_{cat}. A comparison of the k_{cat} values of OTCase in the presence or absence of guanidine gives values of 12 000 ± 2000 min^{-1} or 5.7 ± 0.2 min^{-1}, respectively. This corresponds to a 2000-fold increase in turnover number or 10% of wild-type. The K_M^{CP} values do

Table 2: Rescue of OTCase R57G Mutant by Guanidines and Urea[a]

compound	K_R (mM)[b]	k_{cat} (1/min)[c]	K_M^{CP} (mM)[c]	$k_{cat}^{rescue}/k_{cat}^{no\ rescue}$
guanidine	22 (3)	12000 (2000)	0.6 (0.1)	2000
methylguanidine	130 (40)	1400 (100)	0.28 (0.06)	230
ethylguanidine	100 (30)	680 (150)	12 (3)	120
propylguanidine	25 (4)	110 (40)	4 (2)	18
butylguanidine	16 (3)	80 (10)	5 (2)	14
guanidinoethanol	33 (7)	260 (40)	4.8 (0.8)	43
nitroguanidine	3.1 (1.1)	8 (2)	1.6 (0.7)	1.3
aminoguanidine	28 (3)	2600 (100)	0.35 (0.02)	430
guanylurea	13 (4)	200 (30)	2 (0.4)	33
urea	680 (230)	53 (2)	0.24 (0.04)	9.1
1,3-diaminoguanidine	140 (70)	36 (2)	0.68 (0.04)	6

[a] Kinetic values for k_{cat}, K_M^{CP}, and K_R were determined as described under Materials and Methods. In the absence of guanidine, k_{cat} is 5.7 min^{-1} and K_M^{CP} is 0.22 mM. [b] The value in parentheses is the average error of the mean. [c] The value in parentheses is the standard error of the coefficient.

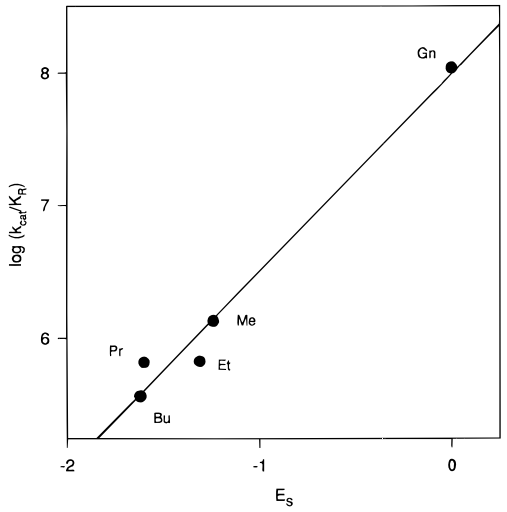

FIGURE 2: Linear regression analysis of steric effect in chemical rescue of OTCase reaction. Data were evaluated for guanidine hydrochloride (Gn), methylguanidine (Me), ethylguanidine (Et), propylguanidine (Pr), and butylguanidine (Bu). Data were fitted to the equation: $\log(k_{cat}/K_R) = \alpha(E_S) + C$.

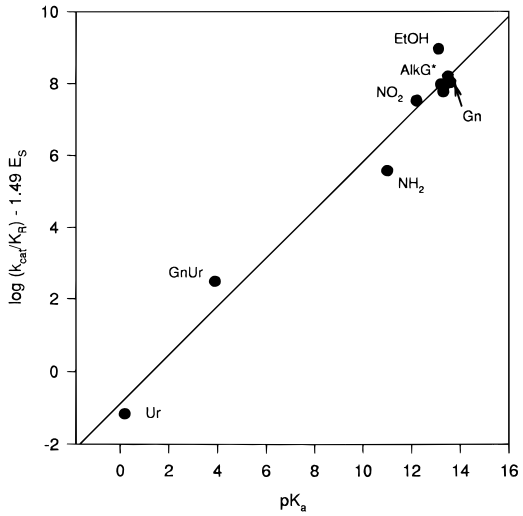

FIGURE 3: Brønsted plot for rate enhancement of OTCase reaction by guanidines. Data were evaluated for guanidine hydrochloride (Gn), aminoguanidine (NH_2), guanylurea (GnUr), guanidinoethanol (EtOH), nitroguanidine (NO_2), and urea (Ur). The data for the alkylguanidines (methyl-, ethyl-, propyl-, and butylguanidine) are clustered at the point designated AlkG*. Data were fitted to the equation: $\log(k_{cat}/K_R) - \alpha(E_S) = \beta(pK_a) + C$.

not change significantly: 0.6 ± 0.1 mM in the presence of guanidine and 0.22 ± 0.01 mM in its absence.

The effects of other guanidines, which were chosen for their variation in size and charge, are shown in Table 2. Urea and guanidine derivatives with nitro and 1,3-diamino substituents exhibit minimal rate enhancement. It should be noted that these guanidines are at the upper extreme in size or lower extreme in pK_a. The reagent showing the greatest degree of chemical rescue is guanidine hydrochloride, which has the largest k_{cat} and a low K_R.

The relationship between steric effects and rate enhancement was evaluated for guanidine and the four alkylguanidines from a plot of log (k_{cat}/K_R) versus E_S (Figure 2). The correlation coefficient for this line is 0.98, and the slope, α, is 1.49 ± 0.13. To assess the effect of charge, data from six other compounds (guanidine, guanidinoethanol, aminoguanidine, nitroguanidine, urea, and guanylurea), ranging in pK_a from 0.2 to 13.6, were plotted as log (k_{cat}/K_R) $- \alpha(E_S)$ versus pK_a (Figure 3). The correlation coefficient for this line is 0.97 with a slope, β, of 0.67 ± 0.04.

The kinetic mechanism of R57G alone or with guanidine added was determined by the inhibition pattern of norvaline, an analog of ornithine, using carbamyl phosphate as the varied substrate. The norvaline inhibition pattern differentiates wild-type OTCase and R57G mutant whereas other substrate inhibition patterns do not differ for the two enzymes. Norvaline is an uncompetitive inhibitor of carbamyl phosphate for the wild-type but becomes noncompetitive in R57G (Kuo et al., 1988). For R57G in saturating levels of guanidine, norvaline is a noncompetitive inhibitor of carbamyl phosphate for the R57G mutant. Thus, guanidine-R57G follows the random kinetic mechanism of the unrescued mutant rather than the ordered Bi−Bi mechanism of the wild-type enzyme.

DISCUSSION

The kinetic data show that the activity of the R57G mutant OTCase can be chemically rescued (partially restored) by the addition of certain guanidines. Measurement of k_{cat} values for different guanidines provides an opportunity to investigate the role of arginine 57 in catalysis. The chemical rescue can be described quantitatively by the equation:

$$\log (k_{cat}/K_R) = \alpha(E_S) + \beta(pK_a) + C$$

where α and β are constants that reflect the magnitude of steric and charge effects, respectively. E_S, the Taft steric parameter, is a variable that describes the overall steric contribution of a given substituent to the reaction rate. The Taft steric parameter was used rather than molecular volume

(Toney & Kirsch, 1989) because E_S better fits the OTCase data.

For the guanidine derivatives examined, the effectiveness of a given guanidine derivative in bringing about chemical rescue shows a strong correlation with the steric parameter. Size and rate enhancement are inversely correlated; i.e., larger substituents produce lower rate enhancement. The poor rescue exhibited by disubstituted guanidines and derivatives with relatively large substituents, e.g., nitroguanidine, may thus be attributed to unfavorable steric effects. In addition, the larger substituents may have to displace water molecules or other ions that might be found in the R57G mutant but not wild-type, active site cavity. For example, Stebbins et al. (1992) studied the ATCase mutant, R54A, where the mutated arginine residue is equivalent to arginine 57 in OTCase. The crystal structure of R54A showed that its active site contains solvent water molecules at positions occupied by arginine side chain nitrogen atoms in the wild-type enzyme. If guanidine binds in R57G OTCase at the site occupied by the arginine 57 side chain, presumably it would replace such water molecules.

When corrections are made for steric effects, a linear trend is observed between pK_a and reaction rate, i.e., that higher pK_a is associated with higher reaction rate. The influence of charge is quantitated in the Brønsted constant. The value of 0.67 for β indicates a role for a positive charge in the rescued transition state. The importance of a positive charge at position 57 in OTCase is consistent with mechanistic studies on the R57H mutant (Goldsmith & Kuo, 1993). In these studies, rate constants vary significantly with the protonation state of the histidine, which has a pK_a of 9.3. When histidine 57 is deprotonated, the k_{cat} value in R57H is comparable to that of the R57G mutant. Protonation of histidine 57 increases the turnover number nearly 20-fold, relative to that of the deprotonated R57H.

A positive Brønsted constant has been cited in other chemical rescue studies as support for a general base catalysis role for the substituted side chain. Toney and Kirsch (1989), in their chemical rescue investigation of a lysine-substituted aspartate aminotransferase mutant, observed a β value of 0.4. This value was interpreted as 40% of a full positive charge on the proton-accepting amide nitrogen during the rate-determining step in the transamination reaction. A similar conclusion was reached in studies involving a lysine→alanine mutant of leucine dehydrogenase, where β was found to be 0.21 (Sekimoto et al., 1993). In studies of human liver alcohol dehydrogenase (Ehrig et al., 1991), a β value of 0.53 was cited as further evidence that histidine 51 acts as a general base catalyst during alcohol oxidation.

Other chemical rescue studies have suggested alternatives to acid−base catalysis in interpreting Brønsted constants. For example, Harpel and Hartman (1994) concluded from their studies on ribulose-1,5-bisphosphate carboxylase/oxygenase that the observed β value of 1 could indicate that lysine 329 assists in the polarization of the substrate, CO_2, in the transition state instead of acting as a general acid−base catalyst. The classical definition of the Brønsted coefficient applies to both proposed roles (Jencks, 1987), and these could not be readily distinguished in the case of lysine 329.

Studies of ATCase suggest that in OTCase, the role of arginine 57 is to stabilize the transition state of the transcarbamylation reaction rather than participating in general base catalysis. The two enzymes share a common substrate, carbamyl phosphate, and are believed to possess analogous reaction mechanisms. The carbamyl phosphate binding domains are homologous in both enzymes, and ATCase active site residues that interact with carbamyl phosphate (Ke et al., 1988) are conserved in OTCase. A proton-transfer mechanism has been proposed for ATCase, in which a proton is transferred from the amino nitrogen of substrate L-aspartate to the phosphate leaving group (Gouaux et al., 1987). Arginine 54 in ATCase has been directly implicated in ionic stabilization of the transition state (Stebbins et al., 1992). The suggested role for arginine 54 in this mechanism is to stabilize the dianionic form of phosphate during the reaction, thus promoting proton transfer and enhancing phosphate as a leaving group. From their ^{31}P-NMR studies comparing wild-type ATCase with a R54A mutant, Stebbins and co-workers show that the transition-state phosphate is dianionic in the wild-type enzyme but monoanionic in an R54A mutant. From these and other observations, they conclude that the 17 000-fold decrease in k_{cat} observed in the mutant reflects a loss of this charge stabilization provided by arginine 54. We have initiated similar ^{31}P-NMR experiments on OTCase. Preliminary data on the R57G OTCase mutant reveal that guanidine produces an increased line width and upfield chemical shift of the signal corresponding to bound carbamyl phosphate when compared with the unrescued mutant enzyme (M. Rynkiewicz and M. F. Roberts, unpublished observations). These data suggest that kinetic rescue by guanidine may be connected with an altered electronic environment of the bound carbamyl phosphate.

In the pH 7−10 range, the R57G OTCase mutant follows a rapid-equilibrium random Bi−Bi kinetic mechanism (Kuo et al., 1988), which does not change with the addition of guanidine (data not shown). At pH 8 and above, OTCase follows a steady-state ordered Bi−Bi mechanism, with carbamyl phosphate bound before ornithine. For both wild-type and R57G, the pH range for maximal activity is similar, between 7.5 and 9.0 (Kuo et al., 1988). The loss of ordered binding in the mutant therefore is not related to a change in the optimum pH of the mutant.

Cleland has suggested that ordered addition of substrates may result from conformational transitions induced by binding of the first substrate that facilitate the binding of the second substrate (Cleland, 1977). Evidence suggests that for OTCase, a carbamyl phosphate-induced change in conformation is directly linked to k_{cat} for the transcarbamylation reaction (Goldsmith & Kuo, 1993). This transition is readily detected in UV difference spectra of wild-type OTCase in the presence of carbamyl phosphate but is absent for the R57G mutant (Kuo et al., 1988). However with saturating levels of guanidine, the addition of carbamyl phosphate to R57G produces UV difference spectra similar to that of wild-type OTCase (Figure 4).

In summary, we have found that certain guanidines can effect chemical rescue in R57G OTCase, and an investigation has been initiated into factors contributing to the observed reaction rate enhancement. The rescue agent with the greatest increase in k_{cat}, relative to the R57G mutant alone, is guanidine hydrochloride. Based on these studies, a model is proposed to explain the chemical rescue of R57G OTCase by guanidine. In this model, the guanidine molecule binds, by virtue of its charge and size, in the site that would otherwise be occupied by arginine 57 in the wild-type. The model further predicts that guanidine mimics this side chain in several respects: (1) contributing a partial positive charge to the transition state; (2) binding to or near carbamyl

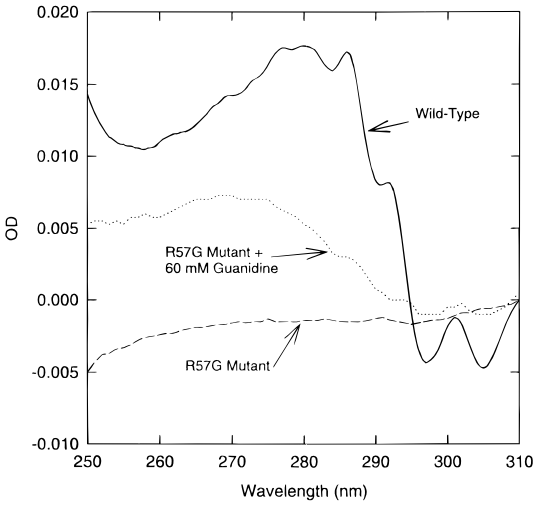

FIGURE 4: UV difference spectra arising from the addition of carbamyl phosphate to wild-type, R57G, or guanidine-rescued R57G OTCase.

phosphate in such a way as to change its electronic environment; (3) participating in the substrate-induced conformational transition required for catalysis. However, guanidine is not a perfect substitute for the arginine 57 side chain as evidenced by the loss of sequential ordered binding and reduction of k_{cat} relative to wild-type. Further kinetic investigations and crystallographic analysis of wild-type, R57G, and guanidine-rescued R57G OTCase (L. Jin, M. Rynkiewicz, J. F. Head, and B. A. Seaton, unpublished observations) are in progress and should help explain these properties.

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