Major Contribution of a Carboxymethyl Group to Transition-State Stabilization by Cytidine Deaminase: Mutation and Rescue†

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ABSTRACT: The crystal structure of an inhibitory complex formed between *Escherichia coli* cytidine deaminase and the transition-state analog 3,4-dihydrouridine indicates the presence of a short H-bond between Glu-104 and the inhibitor. To test the possibility that analogous H-bonds might play a significant role in stabilizing the hydrated substrate in the transition state for deamination, we replaced Glu-104 by alanine. Compared with the wild-type enzyme, the mutant enzyme's affinities for substrate cytidine and product uridine were found to have increased, whereas k_{cat} for deamination of cytidine had been reduced by 8 orders of magnitude. By its presence, the carboxymethyl group of Glu-104 appears to minimize the activation barrier for deamination, not only by stabilizing the altered substrate in the transition state but also by destabilizing the enzyme–substrate and enzyme–product complexes. In the presence of added formate ion, but not in the presence of bulkier carboxylic acids, the low catalytic activity of the mutant enzyme was enhanced substantially.

Cytidine deaminase from *Escherichia coli* is a dimer of identical subunits (31 540 Da/subunit), each containing a single zinc atom (Yang et al., 1992). This enzyme enhances the rate of deamination of cytidine (Figure 1) by a factor of approximately 4×10^{11} (Frick et al., 1987) and binds the transition-state analog inhibitor 3,4-dihydrouridine (**III**, Figure 1) with a dissociation constant of 1.2×10^{-12} M, more than 9 orders of magnitude lower than that of uridine, the substrate for the reverse reaction (Frick et al., 1989). The gene encoding cytidine deaminase has been cloned, its amino acid sequence has been deduced from the nucleotide sequence (Yang et al., 1992), and the role of each active site zinc-liganding residue has been investigated by mutation to alanine (Smith et al., 1994).

To enhance the rate of any reaction, an enzyme must single out for chemical recognition those few features of a substrate that change as it passes from the ground state to the transition state. Structural features of the active site that allow it to make this distinction are therefore of special interest. A tetrahedrally oriented hydroxyl group at C-4 is one conspicuous feature of the suspected hydrated intermediate in deamination (**I**, Figure 1), and of the corresponding inhibitor (**III**, Figure 1), that might enable the enzyme's active site to distinguish these species from the planar substrate and product of the reaction. In accord with this possibility, replacement of the 4-OH group of **III** by hydrogen has been found to reduce this inhibitor's binding affinity by a factor of 2.5×10^7 (Frick et al., 1989). The crystal structure of the enzyme's complex with 3,4-dihydro-5-fluorouridine (**III**) has been solved (Betts et al., 1994), unveiling strong interactions between the 4-OH group of this inhibitor and three functional groups on the enzyme: (1) the backbone amide group of Cys-129, (2) a zinc atom at the active site, and (3) the carboxylate group of Glu-104 (Figure 2).

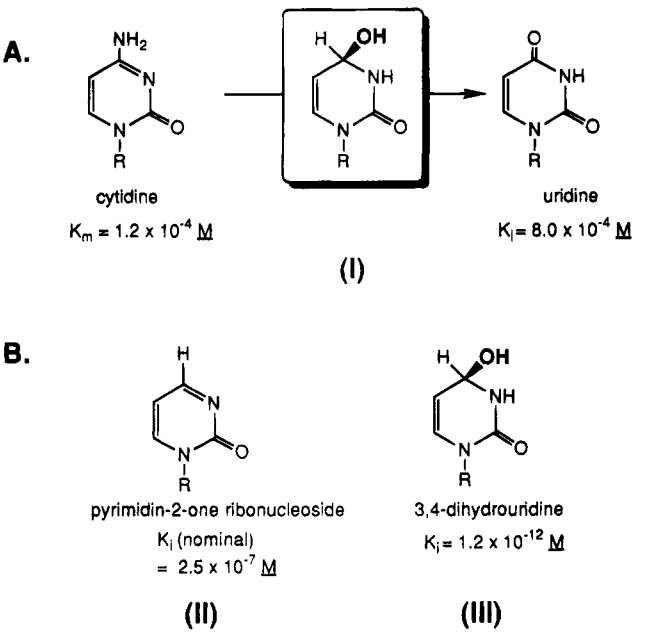

FIGURE 1: (A) Proposed mechanism of action of cytidine deaminase, involving direct water attack on cytidine to generate a tetrahedral intermediate (**I**). (B) Affinities of *E. coli* cytidine deaminase for the ground-state analog inhibitor zebularine (**II**) and the transition-state analog inhibitor 3,4-dihydrouridine (**III**).

These observations led to the inference that the carboxylate group of Glu-104 might play a central role in the catalytic process by serving as a general base, to abstract a proton from the 4-OH group of the tetrahedral intermediate (**I**, Figure 1). After abstraction of that proton, the resulting

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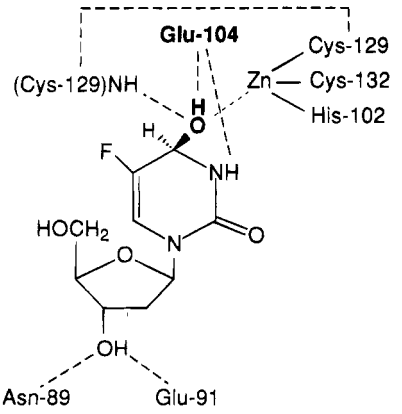

FIGURE 2: Enzyme interactions with a tetrahedral intermediate in deamination of cytidine, inferred from the crystal structure of the inhibitory complex formed between cytidine deaminase and 3,4-dihydrouridine (Betts et al., 1993).

carboxylic acid would be in a position, near the edge of the pyrimidine ring, to furnish a hydrogen atom to ammonia, assisting its departure (Betts et al., 1994).

In the present work, we replaced Glu-104 by alanine, in an attempt to evaluate its role in the action of cytidine deaminase. After observing a profound reduction in catalytic activity, with no loss in the enzyme's affinity for substrates or products, we investigated the possibility that the catalytic activity of the mutant enzyme might be "rescued" by addition of carboxylic acids, as first described in the rescue of activity of a mutant transaminase by Toney and Kirsch (1989).

MATERIALS AND METHODS

Recombinant DNA Procedures. The subcloning and sequencing of *cdd* gene constructs was performed as described by Smith et al. (1994). Genes encoding wild-type and mutant proteins, along with the necessary upstream promoter/operator sequences, were derived from pBR322 plasmids. After mutagenesis of the wild-type *cdd* gene, the region of the mutant gene containing the codon change was verified by nucleotide sequence determination and subcloned as an *Nsi*I−*Bgl*II-ended DNA fragment into unmutagenized and *Nsi*I−*Bgl*II-digested wild-type plasmid pAA5970. This procedure ensured that only the desired mutations were present in the final clone used for enzyme expression.

Enzyme Purification. Plasmids harboring the wild-type *cdd* gene and the E104A mutant allele were transformed into *E. coli* SS6130 (*cytR* Δ*cdd*) for protein production, as described by Smith et al. (1994). In this strain, transcription of the plasmid-borne *cdd* gene is completely derepressed, and cytidine deaminase is expressed only from the plasmid-borne gene. Bacterial cultures were grown for 16−18 h at 37 °C in Luria broth containing ampicillin (100 μg/mL), recovered by centrifugation (3 g wet weight), and suspended in buffer (12 mL) containing Tris-HCl (50 mM, pH 7.5), KCl (50 mM), and glycerol (10%,v/v). Cell extracts were prepared by passage of this material through a French press (11 000 psi). Intact cells and cell debris were removed by centrifugation (15 000 rpm for 15 min in an SS-34 rotor), and the crude supernatant was centrifuged at 100 000*g* for 1 h. The resulting supernatant was then applied to an anion exchange column (Poros II Q, 1 × 8 cm, Perspective Biosystems, Cambridge, MA) equilibrated with the same buffer. At least 90% of the enzyme was recovered in the flow-through fraction, with a 4-fold purification. This fraction was diluted 3-fold with Tris-HCl buffer (50 mM, pH 7.5, containing 10% (v/v) glycerol) and applied to an anion-exchange column (HR 10/10 MONO-Q, Pharmacia, Piscataway, NJ) equilibrated with the same buffer. When the column was developed with a gradient of increasing KCl, cytidine deaminase emerged at a salt concentration of 0.15 M. Analysis of enzyme-containing fractions by SDS−PAGE indicated that the enzyme was at least 95% homogeneous.

Assays of Wild-Type Enzyme. Influences of pH on k_{cat} and K_m were determined with potassium acetate buffers (0.02 M) in the pH range between 4 and 5.5, potassium phosphate buffers (0.02 M) in the pH range between 5.5 and 8, and Tris-HCl buffers (0.02 M) in the pH range between 7.7 and 9, with ionic strength maintained at 0.1 by addition of KCl. The reaction was monitored at 282 nm, by the disappearance of cytidine, using cuvettes maintained at 25 °C, as described by Frick et al. (1989).

HPLC Assay of the Activity of the Mutant Enzyme. Reverse-phase HPLC was used to monitor the slow conversion of cytidine to uridine by the mutant enzyme. Mixtures containing Tris-HCl buffer (50 mM, pH 7.5), mutant enzyme (5×10^{-5} M), and cytidine (1×10^{-6} to 7×10^{-4} M) were incubated at 25 °C. At four or more timed intervals, aliquots (200 μL) were deproteinized by ultrafiltration (Microcon 10, Amicon Corp.) at 14000*g* for 5 min. The filtrate (100 μL) was injected onto a reverse-phase C-18 HPLC column (0.46 × 25 cm, Whatman Partisil 10, ODS-3) and eluted with water (1 mL/min). Cytidine and uridine were well separated, with retention times of 9.2 and 12.5 min, respectively. Elution of substrate and product was monitored at 254 nm, and the integrated peak areas of the substrate and product were compared with standard samples of known concentration. Kinetic constants were obtained by nonlinear regression analysis of the results.

Ligand Binding by the Mutant Enzyme. Dissociation constants of enzyme complexes with [5,6-$^3H_2$]uridine and [5-^{3}H]cytidine (purchased from Moravek Biochemicals, Inc.) were determined by equilibrium dialysis using Spectra Por type 3 membranes (Spectrum Medical Industries, Los Angeles, CA). A solution (100 μl) containing the mutant enzyme (5×10^{-6} M subunits in Tris-HCl buffer, 50 mM, pH 7.5) was added to one chamber of a microequilibrium dialysis apparatus (Bel-Art Corp.), and the second chamber contained a solution (100 μL) of the radioactive ligand in the same buffer, at concentrations ranging from 5×10^{-7} to 1×10^{-4} M. The mixture was allowed to equilibrate at 4 °C for a period of 12 h, during which the cell was rotated at 60 rpm to minimize concentration polarization.

Activation of the E104A Mutant by Formate. Assay mixtures contained cytidine at a concentration well in excess of K_m (1×10^{-3} M), varying concentrations of sodium formate with a pH adjusted to either 4.5 or 7.0 with HCl, and sufficient NaCl to maintain an ionic strength of 5.0. Mutant enzyme was added to produce a final subunit concentration of 5×10^{-5} M in a reaction volume of 3 mL. Aliquots (200 μL) of the reaction mixture were removed at timed intervals over a period of 20 h, analyzed by HPLC as described above, and used to obtain a value for the apparent k_{cat} at that formate concentration. Second-order rate constants were obtained by linear regression analysis of apparent k_{cat} values, plotted as a function of the concentration of formate ion.

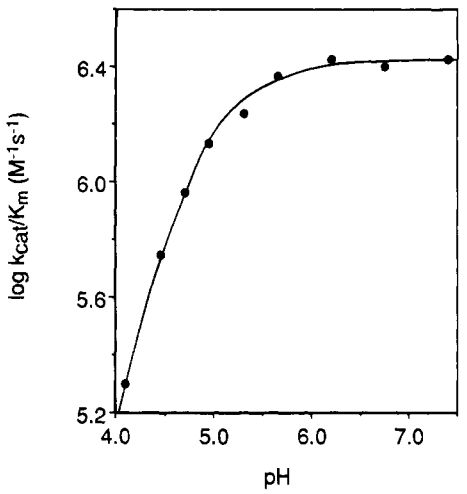

FIGURE 3: Influence of pH on k_{cat}/K_m for wild-type cytidine deaminase (0.06 unit/mL) acting on cytidine, at 25 °C in acetate, phosphate, and Tris-HCl buffers.

Zinc Analysis. Samples of enzyme (0.5−1.0 mg/mL) were analyzed for zinc content using an Instrumentation Laboratories S-12 flame atomic absorption spectrometer, as described previously (Smith et al., 1993).

RESULTS

Influence of pH on k_{cat}/K_m for the Wild-Type Enzyme. Figure 3 shows that the value of k_{cat}/K_m remained invariant at pH values much above pH 5.5, but fell sharply as the pH fell below 5.5. This behavior appears consistent with the presence of two ionizable groups on the free enzyme or the free substrate, both of which must be present in basic form if catalysis is to occur. However, these ionizations occur at pK_a values that appear to be too narrowly separated for their individual values to be determined. The solid line in Figure 3 shows the results expected if a double ionization occurred at $pK_a = 4.2$, with the loss of two protons.

Preparation and Properties of Cytidine Deaminase Mutant E104A. After the site-specific change of Glu-104 to Ala had been introduced into the *E. coli cdd* gene, the presence of the mutation (GAA → GCG) was verified, and the mutant protein was expressed as described earlier for mutants of *E. coli* cytidine deaminase involved in zinc coordination (Smith et al., 1994). As in those mutant proteins, the CD spectrum of the purified E104A mutant protein was indistinguishable from that of the wild-type enzyme. Analysis of wild-type and E104A proteins by matrix-assisted laser desorption mass spectrometry indicated that both proteins contained subunits of approximately 32 000 Da. Using protease V8, an enzyme that cleaves after glutamate residues, only the differences expected between the mutant and wild-type proteins were observed. This evidence suggests that the E104A protein resembles the wild-type protein in its subunit size and folding properties. After exhaustive dialysis against HEPES−HCl buffer (10 mM, pH 7) that had been freed of contaminating metal ions by passage through a Chelex 100 column (Bio-Rad), the mutant protein was found to contain 0.96(±0.05) zinc atoms per subunit (31 540 Da), similar to the zinc content of the wild-type enzyme (Yang et al., 1992).

Effects of Glu-104 Replacement on the Binding and Interconversion of Substrate and Product. Table 1 shows that when alanine replaced Glu-104, the value of k_{cat} was reduced by a factor of approximately 10^8 compared with that of the wild-type enzyme. However, the mutant enzyme's apparent affinity for substrate and product had increased, with a K_m value for cytidine approximately 29-fold lower than that of the wild-type enzyme. The catalytic activity of the mutant enzyme had been reduced to such an extent that the dissociation constant of its complex with isotopically labeled cytidine could be determined by equilibrium dialysis, yielding a K_d value of 6.9×10^{-6} M, in reasonable agreement with its K_m value for cytidine (4.2×10^{-6} M), determined kinetically. In addition to its enhanced affinity for substrate cytidine, the mutant enzyme showed a 118-fold increase in affinity for product uridine, compared with that of the wild-type enzyme (Table 1). The k_{cat} value of the mutant enzyme, although many orders of magnitude smaller than that of the wild-type enzyme, was approximately 4 orders of magnitude larger than the rate constant of the nonenzymatic reaction under similar conditions (Frick et al., 1987).

Table 1: Effect of Glu-104 Replacement on Cytidine Deamination and Uridine Binding

	wild type[a]	E104A[b]
k_{cat} (s^{-1})	299	2.6×10^{-6}
K_m, Cytidine (μM)	120	4.2, 6.9[c]
k_{cat}/K_m (M^{-1} s^{-1})	2.6×10^6	0.62
K_i, uridine (μM)	800	6.8[c]

[a] Smith et al. (1994). [b] This work. [c] True dissociation constants were determined by equilibrium dialysis.

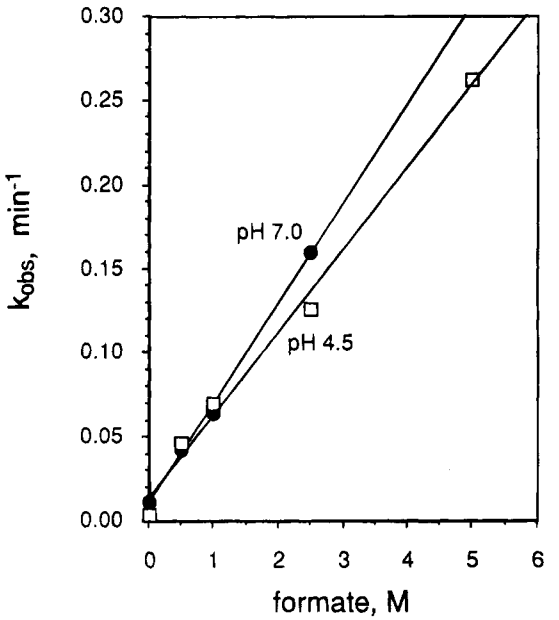

FIGURE 4: Effect of formate on k_{cat} for deamination of cytidine by the mutant enzyme, at 25 °C and ionic strength 5.0, maintained by addition of NaCl. The ratio between the rates observed at pH 4.5 and 7.0 indicates that the formate anion ($pK_a = 3.77$) is the reactive species.

Activation of the E104A Mutant by Addition of Formate. Figure 4 shows that the action of the mutant enzyme on cytidine, at a concentration (10^{-3} M) well in excess of K_m, was greatly enhanced in the presence of sodium formate. Under these conditions, k_{cat} rose linearly with increasing concentration of formate at both pH 7.0 and 4.5. No evidence of saturation was observed at formate concentrations up to 5 M. The catalytic rate constant, $k_{formate}$, obtained from the slopes of these lines, was 3.64×10^{-5} M^{-1} s^{-1} at pH 7.0 and 3.30×10^{-5} M^{-1} s^{-1} at pH 4.5. This behavior

is consistent with a dependence of the rate on the concentration of the anionic form of formate rather than its conjugate acid.

Attempts to activate the mutant enzyme with 1 M solutions (containing 50 mM Tris-HCl, pH 7.0) of sodium phosphate, sodium carbonate, sodium borate, sodium trifluoroacetate, sodium difluoroacetate, sodium monofluoroacetate, and sodium acetate, adjusted to pH 7.0, showed no detectable rate enhancement. Sodium formate at a concentration of 5 M (the highest concentration used in the rescue experiments described above) produced no detectable deamination of cytidine in the absence of protein, during the time period of these experiments, nor did high concentrations of sodium formate significantly affect catalysis by the wild-type enzyme.

DISCUSSION

The dependence of k_{cat}/K_m on pH (Figure 3) is consistent with a requirement that two functional groups of the enzyme or substrate, with pK_a values lower than 5, must both be present in basic form if catalysis is to occur. One of these ionizations probably involves the conjugate acid of cytidine (pK_a = 4.2): in the case of fungal adenosine deaminase (Sharpless et al., 1967), the substrate for deamination must be present as the neutral species for productive binding to occur. The second ionization may involve the carboxylate group of Glu-104. The detailed structure of the enzyme's complex with a transition-state analog inhibitor (Betts et al., 1994), in conjunction with the extreme importance of this residue for catalytic activity as discussed below, suggests that Glu-104 might serve as a general base, abstracting a proton from the 4-OH group of the tetrahedral intermediate (**I**, Figure 1).

When Glu-104 was replaced by alanine, the k_{cat}/K_m value of the mutant enzyme was reduced by a factor of 4.2×10^6. This value, along with major reductions in catalytic efficiency that result from single amino acid substitutions in other enzymes[1] (Table 2), seems consistent with a major role for these residues in catalysis. The conformation of the present mutant enzyme appears to resemble that of the wild-type enzyme, as judged from its behavior on purification, its CD spectrum, its zinc content, and its high affinity for substrates, products, and inhibitors of the reaction. In the crystal structure of the enzyme's complex with the transition-state analog inhibitor **III**, the carboxylate oxygen atoms of Glu-104 are in a position to form two short H-bonds, one with the 4-OH group of **III** (2.5 Å) and the other with N-3 of the pyrimidine ring (2.7 Å) (Betts et al., 1994). In nonaqueous environments, the strengths of short H-bonds are so great that the existence of two such bonds could suffice to explain the observed difference in catalytic efficiency between the wild-type and mutant enzymes (Wolfenden and Kati, 1991).

Table 2: Some Contributions of Single Amino Acid Residues to Transition-State Stabilization, Inferred from the Results of Singe-Site Mutations

enzyme	substitution	$\Delta\Delta G^a$
cytidine deaminase[b]	Glu-104 → Ala	−8.8
adenylate kinase[c]	Arg-138 → Met	−7.0
carboxypeptidase A[d]	Arg-127 → Met	−6.0
valyl-tRNA synthetase[e]	Thr-52 → Ala	−6.0
methionyl-tRNA synthetase[f]	Tyr-359 → Ala	−5.2
adenosine deaminase[g]	Glu-217 → Ala	−5.2

[a] $\Delta\Delta G = -RT \ln[k_{cat}/K_m(\text{mutant})/k_{cat}/K_m(\text{wild type})]$. [b] This work. [c] Yan et al. (1990). [d] Phillips et al. (1990). [e] Borgford et al. (1987). [f] Ghosh et al. (1991). [g] Bhaumik et al. (1993).

Somewhat surprisingly, the affinity of the mutant enzyme for product uridine was found to have increased 118-fold compared with that of the wild-type enzyme, and the mutant enzyme's apparent affinity for substrate cytidine had increased by a factor of 29 compared with that of the wild type enzyme (Table 1). Cytidine deamination was retarded to such an extent in the mutant enzyme that cytidine's dissociation constant could be determined by independent binding experiments using radiolabeled cytidine, yielding a value similar to the K_m value for cytidine. Thus, although conversion of Glu-104 to alanine was found to have reduced the catalytic efficiency of the enzyme by many orders of magnitude, its affinities for substrate cytidine and product uridine had *increased* considerably compared with the apparent affinities of the wild-type enzyme for substrate and product. By its presence, the carboxymethyl group of Glu-104 appears to minimize the activation barrier for deamination, not only by stabilizing the altered substrate in the transition state for deamination but also by destabilizing the enzyme−substrate and enzyme−product complexes (Figure 5).

Addition of sodium formate, but not addition of bulkier carboxylic acids or inorganic phosphate, resulted in substantial increases in the deaminase activity of the mutant enzyme. In their pioneering experiments on catalytic rescue, Toney and Kirsch (1989) observed that the activity of a mutant aspartate aminotransferase was highly sensitive to the molecular volume of the rescuing amine. The comparable magnitudes of the effects of formate observed at pH 7.0 and at 4.5 (Figure 4) are consistent with a catalytic role for the anionic species of formic acid, and not for its conjugate acid. Comparison of the second-order rate constant at pH 7.0 (3.6×10^{-5} M^{-1} s^{-1}) with k_{cat} for the wild-type enzyme, 299 s^{-1}, suggests the advantage that is gained by restricting the rotational and translational motions of the carboxymethyl group of Glu-104. The concentration of formate ion that would be required to render the activity of the mutant enzyme equivalent to that of the wild-type enzyme would be 8.2×10^6 M.

Since formate ion produced no significant enhancement of the reaction rate in the absence of enzyme under otherwise similar conditions, the formate-assisted reaction seems likely to occur at the mutant enzyme's active site. From the observation that the mutant enzyme binds substrate and product with even greater affinity than does the wild-type enzyme, it seems reasonable to infer that mutation has not

[1] Table 2 is arbitrarily limited to those cases in which it appears to have been clearly established that truncation of a single active site residue of an enzyme reduces its value of k_{cat}/K_m by a factor of 5000 or more, equivalent to an increase of 5 kcal/mol in free energy of activation. This list does not pretend to be exhaustive, and the authors apologize for errors of omission that have doubtless been made. Replacement of a single amino acid has sometimes been reported to result in "complete" elimination of the catalytic activity of an enzyme, without mention of the upper limit of activity that would have been detectable under the experimental conditions used to measure activity. Experimental obstacles to measuring very low activities may, of course, be insurmountable in those cases in which the uncatalyzed reaction proceeds at an appreciable rate. Also not included in this table are many hundreds of single-site mutations of enzymes that have been reported to lead to relatively minor losses of activity.

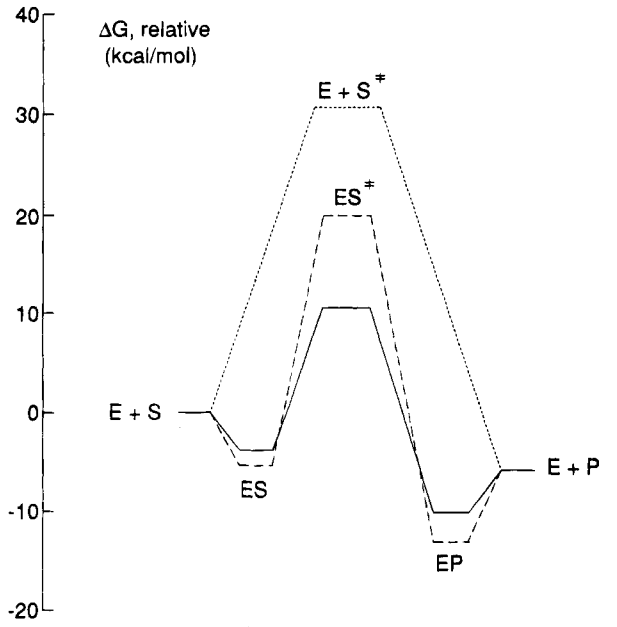

FIGURE 5: Free energy profile for the interconversion of cytidine (S) and uridine (P) in the absence of enzyme [dotted line, from Frick et al. (1987)], by the E104A variant (dashed line), or by the wild-type enzyme [solid line, from Smith et al. (1994)].

seriously disrupted the structural integrity of the active site. The inability of acetate, fluoroacetate, difluoroacetate, trifluoroacetate, carbonate, or inorganic phosphate to rescue the activity of the mutant enzyme seems understandable in terms of a mechanism involving formate replacement of the missing carboxymethyl group of glutamate, considering the space-filling requirements of these larger acids. Even the acetate ion might not fit easily into the cavity created by conversion of glutamate to alanine, because of the expected overlap between the methyl hydrogen atoms of acetate and the methyl hydrogen atoms of alanine.

The mere observation that formate ion rescues the activity of the mutant enzyme does not, by itself, establish the mechanism by which rescue occurs. In the native enzyme, the side chain of Glu-104 projects into the "bottom" of the active site pocket, in such a position that it appears to be occluded completely by bound nucleosides from contact with bulk solvent. Indeed, when the enzyme binds a transition-state analog inhibitor, the analog is so closely sequestered that less than 0.2% of its surface is accessible to solvent water (Betts et al., 1994). In view of this tight fit, it seems unlikely that formate ion could assist the reaction by "leaking" into the active site after the substrate is bound. Instead, rescue may depend on the chance presence of formate within the active site before substrate cytidine is bound and its orientation in a position appropriate for catalysis. In view of those constraints, it seems understandable that rescue is a rare event.

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