

# Residues Involved in the Mechanism of the Bifunctional Methylenetetrahydrofolate Dehydrogenase-Cyclohydrolase

THE ROLES OF GLUTAMINE 100 AND ASPARTATE 125\*

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**The human bifunctional dehydrogenase-cyclohydrolase domain catalyzes the interconversion of 5,10-methylene- $H_4$ folate and 10-formyl- $H_4$ folate. Although previous structure and mutagenesis studies indicated the importance of lysine 56 in cyclohydrolase catalysis, the role of several surrounding residues had not been explored. In addition to further defining the role of lysine 56, the work presented in this study explores the functions of glutamine 100 and aspartate 125 through the use of site-directed mutagenesis and chemical modification. Mutants at position 100 are inactive with respect to cyclohydrolase activity while preserving significant dehydrogenase levels. We succeeded in producing a K56Q/Q100K double mutant, which has no cyclohydrolase yet retains more than two-thirds of wild type dehydrogenase activity. Neither activity is detectable in aspartate 125 mutants with the exception of D125E. The results indicate that the function of glutamine 100 is to activate lysine 56 for cyclohydrolase catalysis and that aspartate 125 is involved in the binding of the  $H_4$ folate substrates. In highlighting the importance of these residues, catalytic mechanisms are proposed for both activities as well as an explanation for the differences in channeling efficiency in the forward and reverse directions.**

The human bifunctional dehydrogenase-cyclohydrolase domain (DC301)<sup>1</sup> of the human NADP<sup>+</sup>-dependent trifunctional methylene- $H_4$ folate dehydrogenase/methenyl- $H_4$ folate cyclohydrolase/formyl- $H_4$ folate synthetase catalyzes two sequential reactions involved in the interconversion of substituted tetrahydrofolates (Scheme I). The interconversion of 5,10-methylene- $H_4$ folate with 5,10-methenyl- $H_4$ folate is accomplished through the NADP<sup>+</sup>-dependent dehydrogenase activity, whereas 5,10-methenyl- $H_4$ folate and 10-formyl- $H_4$ folate are interconverted by the cyclohydrolase activity. A significant amount of the labile methenyl- $H_4$ folate intermediate is channeled between the two activities in the forward direction (50–60%), whereas in the reverse direction, channeling is complete (1). Because 5,10-methylene- $H_4$ folate and 10-formyl- $H_4$ folate are important contributors to methionine, serine, thymidylate, and purine syntheses (reviewed in Ref. 2), their interconversion

within eukaryotic and prokaryotic cells is crucial to maintain a proper balance of one-carbon precursors.

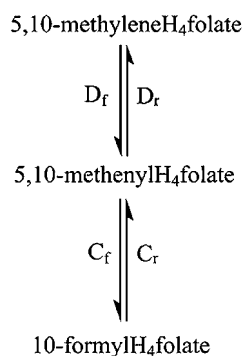
Several lines of evidence have pointed to the fact that both these activities share a common active site. Proteolysis experiments that isolated the bifunctional domain from the trifunctional enzyme failed to resolve the dehydrogenase from the cyclohydrolase activities (3, 4). Chemical modification of the enzyme with DEPC, phenylglyoxal, and carbodiimide-activated folate showed that both activities could be simultaneously inactivated and that DEPC and phenylglyoxal modification could be protected with folate and NADP<sup>+</sup> (5–7). The kinetic parameters for the cyclohydrolase activity are affected by NADP<sup>+</sup> and 2',5'-ADP (1, 8). Finally, the covalent incorporation of 1 mol of <sup>3</sup>H-folate/mol of enzyme (6) and equilibrium dialysis studies (7, 8) further indicated that only one NADP<sup>+</sup> and one tetrahydrofolate binding site exist per monomer of the dimeric enzyme.

The crystal structure of DC301 with bound NADP<sup>+</sup> revealed that each subunit of the homodimer consists of two domains connected by two long  $\alpha$ -helices, creating a single cleft between them. The relative positions of the two domains is different in each subunit of the dimer, indicating that these domains can move around two well defined hinge regions and adapt the size of the cleft to accommodate substrates (9). NADP<sup>+</sup> binds to the C-terminal domain on one side of the active site cleft through interactions of the 2'-phosphate with Arg<sup>173</sup> and Ser<sup>197</sup> (Fig. 1). Mutagenesis studies confirmed the importance of these two residues in dinucleotide cofactor binding (10). The structure of DC301 with bound folate analogs localized the folate binding site to the other side of the cleft (11) and involved a conserved YXXXX motif. Tyr<sup>52</sup> was found to only contribute to substrate positioning through hydrophobic stacking interactions, whereas Lys<sup>56</sup> mutants completely lost cyclohydrolase activity and retained residual dehydrogenase activity. From these structures and existing mutagenesis data, a preliminary mechanism was proposed for DC301 catalysis in the forward direction (11) and revealed several other substructures that might be involved in the function of this enzyme. A Ser<sup>49</sup>-Gln<sup>100</sup>-Pro<sup>102</sup> motif was suggested to be important in catalysis, because the three residues apparently coordinate the binding of a water molecule and Gln<sup>100</sup> makes a hydrogen bond contact with the catalytic lysine. Moreover, the structure of DC301 with the bound Ly345899 inhibitor suggests a role for Asp<sup>125</sup> in folate binding (Fig. 1b). A flexible loop spanning positions 241–250 positioned at roughly 10 Å from the active site may be involved in closing over the active site upon substrate binding and protecting the labile methenyl- $H_4$ folate intermediate from release to the solvent. Through site-directed mutagenesis and chemical modification, this study probes the roles of Gln<sup>100</sup>, Asp<sup>125</sup>, and the flexible loop (residues 241–150) in substrate binding, channeling, and catalysis.

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<sup>1</sup> The abbreviations used are: DC301, dehydrogenase-cyclohydrolase domain; D<sub>f</sub>, forward dehydrogenase; C<sub>f</sub>, forward cyclohydrolase;  $H_4$ folate, tetrahydrofolate; DEPC, diethylpyrocarbonate.



SCHEME 1. Reactions catalyzed by methylene-H<sub>4</sub>folate dehydrogenase and methenyl-H<sub>4</sub>folate cyclohydrolase.

#### EXPERIMENTAL PROCEDURES

**Materials**—(*R,S*)-methenyl-H<sub>4</sub>folate was purchased from B. Schircks Laboratories (Jona, Switzerland). (*R,S*)-H<sub>4</sub>folate was synthesized according to the protocol by Drury *et al.* (12) and stored in sealed glass vials at 4 °C. [<sup>2</sup>H]Formaldehyde was obtained from Cambridge Isotope Laboratories, Inc. Diethylpyrocarbonate was purchased from Sigma. 2',5'-ADP-Sepharose 4B was obtained from Amersham Biosciences. All other chemicals were of analytical grade.

**Site-directed Mutagenesis of DC301**—Mutations to pBKe-DC301 (10, 13, 14) were introduced using the *in vitro* overlap extension method of PCR mutagenesis (15). PCR primers were synthesized by the Sheldon Biotechnology Center, McGill University. PCR amplification was performed using a Progene (Techne) thermocycler in a buffer containing 10 mM KCl, 20 mM Tris-HCl, pH 8.8 at 25 °C, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3 mM MgSO<sub>4</sub>, 0.1% Triton X-100, and 0.1 mg/ml bovine serum albumin. Template DNA (5.4 ng) was used with 10 pmol of each primer, 125 μM of each dNTP, and 1 unit of Vent<sup>®</sup> polymerase (New England Biolabs) in a 50-μl reaction mix. The samples were then subjected to 30 cycles of PCR: 30 s at 94 °C (denaturation), 30 s at 55 °C (annealing), and 30 s at 72 °C (extension). The products of the first two PCR reactions using sense-mutagenic primer with antisense 3'-flanking oligomer and antisense-mutagenic primer with sense 5'-flanking oligomer were identified by electrophoresis and purified. A sample of each product (~2.7 ng) was used as template in a third PCR reaction with the 5'- and 3'-flanking oligo primers. The product from this reaction was digested with appropriate restriction endonucleases (New England Biolabs) and ligated into wild type pBKe-DC301 digested with the same enzymes. Clones were initially screened for the presence of an introduced restriction site, and the entire fragment was sequenced to confirm the integrity of each mutant (Sheldon Biotechnology Center, McGill University and Canadian Molecular Research Services Inc.).

**Restoration of the Hexahistidine Tag on the C terminus of DC301**—Following the original construction of a hexahistidine tag at the C terminus of DC301, the construct was found to be truncated (10), resulting in a trihistidine tag. Although purification was still possible at lower concentrations of imidazole, purification of the enzyme from this construct by Ni-NTA agarose chromatography was not uniformly successful as judged by SDS-PAGE analysis. To improve the ease of purification, nucleotides encoding an additional three histidine residues were added to the wild type DC301 construct by PCR-mediated mutagenesis as described above. The enzyme from this construct eluted from the Ni-NTA column at a much higher concentration of imidazole (150 mM) and was consistently of high purity (estimated at >95%) with the expected specific activities for both D<sub>f</sub> and cyclohydrolase. All subsequent DC301 mutants were produced through PCR-mediated mutagenesis and subcloned into this hexahistidine construct for expression.

**Protein Expression and Purification**—Constructs were transformed into *Escherichia coli* BL21 cells (Stratagene) and expressed as described previously (13) with the exception that the final concentration of isopropyl-1-thio-β-D-galactopyranoside was 2 mM. The purification of recombinant protein essentially followed the method described earlier (11) with modifications. The wash steps involved washing the Ni-NTA column three times with 10 column volumes of buffer A (0.1 M potassium phosphate, pH 7.3, 20% (v/v) glycerol, 0.3 M NaCl, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamide) containing 5, 25, and 35 mM imidazole, respectively. Proteins were eluted with 10 column volumes of buffer A containing 36 mM 2-mercaptoethanol and 150 mM imidazole. Fractions containing purified proteins

were identified using the forward dehydrogenase (D<sub>f</sub>) assay with the exception of Asp<sup>125</sup> mutants, which were identified using the Bradford protein assay (16). Protein concentrations were determined with this assay using bovine serum albumin as a standard. Purity was assessed by SDS-PAGE (17), and all histidine-tagged proteins were purified to apparent homogeneity. The binding of proteins to 2',5'-ADP-Sepharose was carried out as described previously (10) and monitored by protein assay.

**Enzyme Assays and Kinetics**—All spectrophotometric measurements were performed on a Beckman DU-640 spectrophotometer. Concentrations of dinucleotide and methenyl-H<sub>4</sub>folate were determined using the following extinction coefficients: NADP ε<sub>260</sub> = 18 mM<sup>-1</sup>cm<sup>-1</sup> (18, 19); methenyl-H<sub>4</sub>folate ε<sub>350</sub> = 24.9 mM<sup>-1</sup>cm<sup>-1</sup>. Enzyme assays were performed at 30 °C, and all reported values are the results of at least three separate experiments performed in triplicate.

Unless otherwise noted, enzymatic assays for the D<sub>f</sub> and cyclohydrolase forward reactions and substrate channeling were performed as described previously (1). Standard conditions are highly saturating for both dehydrogenase substrates. For kinetic characterization of D<sub>f</sub>, limiting substrate concentrations were varied from 0.5 to 10 times K<sub>m</sub>. Although previous characterization involved the standard fixed points assay, we used a different approach in this study because the K<sub>m</sub> values were low. Initial rates were instead measured continuously by following the accumulation of 5,10-methenyl-H<sub>4</sub>folate. It was previously determined that neither the ratio of cyclohydrolase to dehydrogenase activities nor substrate concentration affect the extent of substrate channeling (10). Because of its continuous nature, this assay provides for a more accurate determination of low values of K<sub>m</sub> while allowing a maximum of 10% of the limiting substrate to be converted to product.

**Chemical Modification with Diethylpyrocarbonate**—Chemical modification was achieved following a previously described protocol (5) with some modifications. Reactions were performed at 4 °C in a buffer containing 0.1 M potassium phosphate, pH 7.3, 20% (v/v) glycerol, and 0.3 M NaCl. Enzymes were diluted to a concentration of 270 nM and reacted with 380 μM DEPC. DEPC was added from a stock solution prepared in ethanol such that the final concentration of ethanol in the reaction did not exceed 4% (v/v). Reactions were terminated after 0, 1, 3, 5, 7, and 9 min by withdrawing suitable samples directly into dehydrogenase assay mix (see above) and measuring D<sub>f</sub> activity.

#### RESULTS

**Mutations at the 241–250 Flexible Loop**—The presence of a flexible loop located 10 Å over the active site in this enzyme raises certain questions as to its function, especially with regards to substrate channeling. Four mutations were made in this region: Y240A, R250A, Loop3G (replacing three residues, Lys<sup>245</sup>, Lys<sup>246</sup>, and Pro<sup>247</sup> with a glycine), and Loop5G (replacing five residues, Asp<sup>244</sup>, Lys<sup>245</sup>, Lys<sup>246</sup>, Pro<sup>247</sup>, and Asn<sup>248</sup> with a glycine). All loop mutants retained significant amounts of D<sub>f</sub> (from 50 to 80% wild type) and C<sub>f</sub> (from 30 to 70% wild type) activities, indicating that the loop is not critical for catalysis (Table I). Furthermore, K<sub>m</sub> values for all three substrates (methylene-H<sub>4</sub>folate, NADP, and methenyl-H<sub>4</sub>folate) were essentially unchanged compared with the wild type enzyme. When the D<sub>f</sub> activity was measured with [*methylene*-<sup>2</sup>H](*R,S*)-5,10-methylene-H<sub>4</sub>folate, the results indicated that in all mutants hydride transfer is still rate-limiting although the magnitude of the deuterium isotope effect is slightly reduced (3.5 for wild type compared with the loop mutants ranging from 2.44 to 2.85). The channeling of the methenyl-H<sub>4</sub>folate intermediate was essentially unaffected for the loop mutants.

**Mutations at Glutamine 100**—The crystal structure of DC301 revealed the presence of a Ser<sup>49</sup>-Gln<sup>100</sup>-Pro<sup>102</sup> motif that was thought to be important in catalysis (9). Site-directed mutagenesis of serine 49 by Schmidt *et al.* (11) did not reveal any catalytic function for this residue. However, because glutamine 100 is not only absolutely conserved but also forms a hydrogen bond with the catalytic lysine 56, its potential contribution to catalysis was of interest. The role of glutamine 100 was determined by mutation to alanine, asparagine, glutamate, lysine, and methionine. Significant residual D<sub>f</sub> activity was once again detected in all mutants; however, their de-

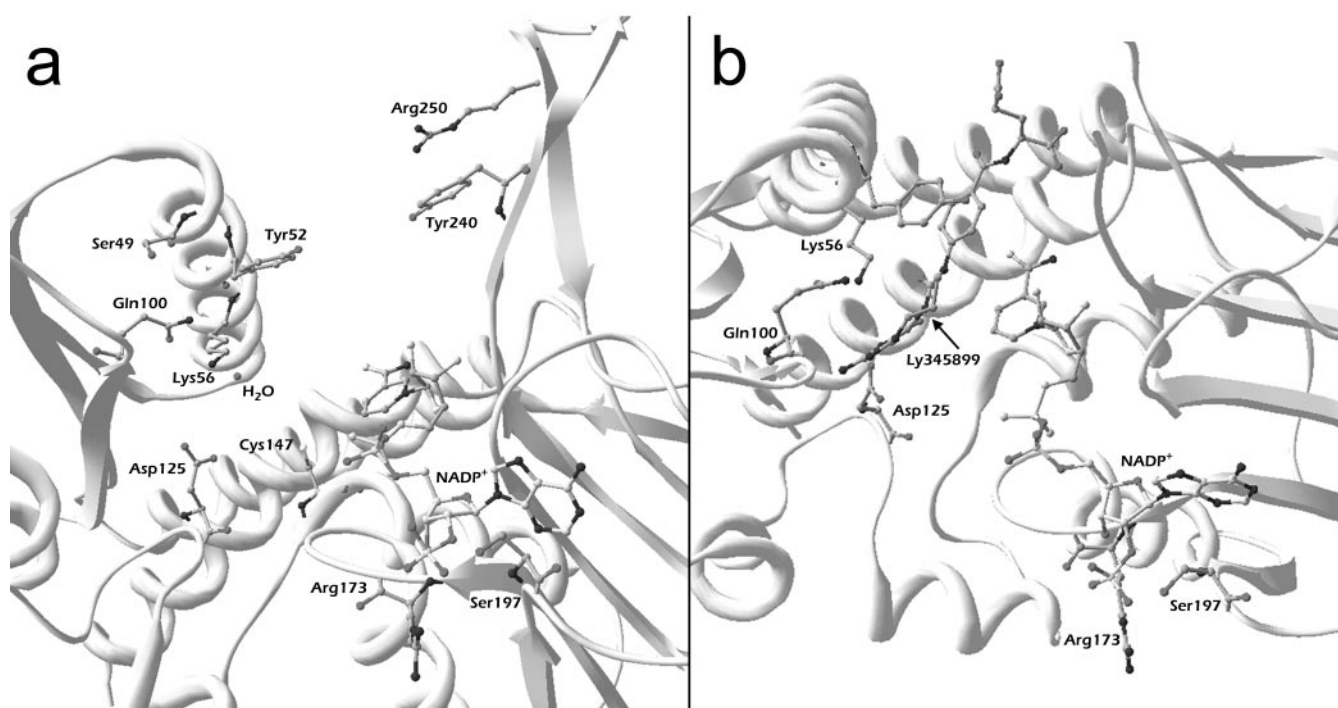


FIG. 1. **Three-dimensional structures.** *a*, the putative folate binding site and the flexible loop structure (residues 241–250). Glutamine 100, lysine 56, and aspartate 125 are shown as well as previously mutated residues and the water molecule whose position is coordinated by glutamine 100 and lysine 56 (9). *b*, active site showing folate analog Ly345899 and NADP<sup>+</sup>. Figures were produced using Protein Data Bank identification number 1A4I with Swiss Protein Database Viewer version 3.6 (30).

TABLE I  
Kinetic properties of Y240A, R250A, Loop3G and Loop5G mutants compared to wild type enzyme

WT, wild type.					
Enzyme	Specific activity D <sub>f</sub>		K <sub>m</sub> methylene-H <sub>4</sub> folate	K <sub>m</sub> NADP μM	v <sub>H</sub> /v <sub>D</sub>
	μmol/min/mg	% WT	μM	μM	
WT	22.4 ± 2.3	100	5.3 ± 1.4	12.4 ± 7.4	3.4 ± 0.4
Y240A	11.4 ± 1.0	50.9	5.7 ± 1.0	15.8 ± 3.6	2.9 ± 0.3
R250A	11.0 ± 2.0	49.4	4.1 ± 0.1	16.8 ± 5.3	2.5 ± 0.3
Loop3G	18.2 ± 1.9	81.1	3.4 ± 0.1	14.2 ± 3.6	2.4 ± 0.1
Loop5G	15.0 ± 1.6	67.0	4.4 ± 1.9	24.8 ± 4.6	2.6 ± 0.4
Enzyme	Specific activity C <sub>f</sub>		K <sub>m</sub> methenyl-H <sub>4</sub> folate	Channeling	
	μmol/min/mg	% WT	μM	%	
WT	161 ± 31	100	60.0 ± 23.4	64.9 ± 3.6	
Y240A	76.5 ± 4.1	47.5	71.7 ± 18.2	49.9 ± 0.7	
R250A	113 ± 11	70.3	49.8 ± 13.5	64.5 ± 0.7	
Loop3G	51.0 ± 2.5	31.7	77.3 ± 21.3	56.3 ± 0.6	
Loop5G	66.7 ± 3.8	41.4	70.5 ± 9.7	63.3 ± 0.7	

crease from the wild type enzyme was more pronounced than with the loop mutants, ranging from 3-fold (Q100N) to 12-fold (Q100K). In contrast, although there is a decrease in D<sub>f</sub>, there is an increase in affinity for methylene-H<sub>4</sub>folate for all Gln<sup>100</sup> mutants, with the exception of Q100K (Table II). K<sub>m</sub> values for this substrate were seen to decrease up to 17-fold (Q100M), whereas the K<sub>m</sub> for NADP remained essentially unchanged. Another interesting finding came with respect to the deuterium isotope effect. When Gln<sup>100</sup> was changed to non-polar (alanine and methionine) or smaller residues (asparagine), hydride transfer became less rate-limiting, suggesting that another step presumably the activation of the folate was now rate-limiting. All mutants at position 100 completely lost C<sub>f</sub> activity within the limits of the assay (<0.5% wild type), indicating that this residue, like lysine 56, is critical for cyclohydrolase catalysis.

**Mutations at Aspartate 125**—The structure of DC301 with bound LY345899 inhibitor showed aspartate 125 making a

hydrogen bond with the C<sub>2</sub>-amino group of the inhibitor (11). Therefore, its role as a potential binding residue for the folate substrates was investigated through site-directed mutagenesis of aspartate 125 to alanine, asparagine, glutamate, methionine, glutamine, and cysteine. With the exception of D125E, all mutants completely lost both D<sub>f</sub> and C<sub>f</sub> activities within the limits of both assays (<0.5% wild type, Table II). Wild type DC301 binds very strongly to 2',5'-ADP-Sepharose and cannot be eluted with up to 2 M phosphate buffer (13). These mutants also bind to 2',5'-ADP-Sepharose affinity columns in a similar manner to the wild type enzyme, indicating that their NADP<sup>+</sup> binding site is not altered (data not shown).

In contrast, D125E retained 90% D<sub>f</sub> activity and 5% C<sub>f</sub> activity, indicating not only that a carboxyl group is required at position 125 but also (as is the case with Gln<sup>100</sup> and Lys<sup>56</sup>) the cyclohydrolase activity is more sensitive than the dehydrogenase to alterations at this position. Although K<sub>m</sub> values were not determinable for most mutants at Asp<sup>125</sup> because of low activ-

TABLE II  
 Kinetic properties of glutamine 100 and aspartate 125 mutants compared to wild type enzyme

Enzyme	Specific activity $D_f$		$K_m$	$K_m$	$v_H/v_D$	Specific activity $C_f$
	$\mu\text{mol}/\text{min}/\text{mg}$	% WT	methylene- $H_4$ folate $\mu\text{M}$	NADP $\mu\text{M}$		
WT	22.4 ± 2.3	100.0	5.4 ± 1.4	12.5 ± 7.5	3.4 ± 0.4	161 ± 31
Q100A	5.1 ± 0.2	22.8	0.35 ± 0.03	3.5 ± 1.4	2.1 ± 0.1	ND <sup>a</sup>
Q100N	7.8 ± 0.6	35.2	0.49 ± 0.02	7.4 ± 2.6	1.8 ± 0.1	ND
Q100E	7.4 ± 1.1	33.0	0.94 ± 0.18	4.6 ± 3.3	2.6 ± 0.5	ND
Q100K	2.4 ± 0.1	10.6	3.7 ± 0.27	12.2 ± 4.5	4.3 ± 0.2	ND
Q100M	4.6 ± 0.3	20.4	0.31 ± 0.08	14.1 ± 2.3	1.5 ± 0.1	ND
K56Q-Q100K	15.3 ± 1.5	68.1	3.6 ± 1.01	4.0 ± 1.2	4.1 ± 0.9	ND
D125A	ND <sup>a</sup>	—	—	—	—	ND <sup>a</sup>
D125N	ND	—	—	—	—	ND
D125E	20.7 ± 1.5	92.2	4.7 ± 0.9	9.7 ± 2.0	2.7 ± 0.9	7.6 ± 0.5 <sup>b</sup>
D125M	ND	—	—	—	—	ND
D125Q	ND	—	—	—	—	ND
D125C	ND	—	—	—	—	ND

<sup>a</sup> ND no detectable cyclohydrolase activity (<0.5% wild type).

<sup>b</sup>  $K_m$  (methyl- $H_4$  folate) for D125E = 117 ± 26  $\mu\text{M}$ .

ity, D125E showed normal  $K_m$  values for the  $D_f$  substrates and only a 2-fold increase in  $K_m$  for methenyl- $H_4$ folate (117  $\mu\text{M}$ ). Because Asp<sup>125</sup> is very far removed (>8 Å) from the methenyl group of the methenyl- $H_4$ folate intermediate based on the structures with folate inhibitors, we propose that its role is in substrate positioning rather than in catalysis in the cyclohydrolase activity.

**Construction and Analysis of a K56Q/Q100K Double Mutant**—Given that K56Q and Q100K have no detectable cyclohydrolase activity but significant dehydrogenase activity (50 and 10% wild type, respectively), it was of interest to find out the properties of the K56Q/Q100K double mutant. DC301 was engineered to essentially interchange the residues at positions 56 and 100, thereby preserving the general environment that these two residues provide while removing any specific interactions they might make in the wild type enzyme. Although  $K_m$  values and the deuterium isotope effect were essentially unchanged from the wild type enzyme, the specific activity for dehydrogenase was restored to two-thirds of wild type, whereas cyclohydrolase activity once again remained undetectable (Table II).

**Effect of DEPC Modification on Forward Dehydrogenase Activity**—Smith and MacKenzie (5) showed in the porcine trifunctional enzyme that both  $D_f$  and  $C_f$  activities could be coincidentally inactivated with DEPC and protected with folate and NADP<sup>+</sup>. Moreover, the rate of the inactivation reaction suggested that a histidine moiety was being modified. Because there are no histidine residues in the putative folate binding site of DC301, we postulated that inactivation might actually have involved lysine 56. Equal concentrations of wild type DC301, K56Q, Q100A, and K56Q/Q100K were treated with 380  $\mu\text{M}$  DEPC over a time course (Fig. 2). The results indicate that the time required to achieve 50% inactivation of  $D_f$  activity is 5-fold greater for K56Q and K56Q/Q100K (20 and 21 min, respectively), and 3-fold greater for Q100A (13 min). The wild type enzyme is protected from inactivation by the presence of folate and NADP<sup>+</sup> as observed previously (5), but no protective effect was apparent with the K56Q mutant (data not shown)

#### DISCUSSION

**The Role of Glutamine 100**—The mutants at glutamine 100 retain significant residual dehydrogenase activity. When mutated to residues that do not donate electrons such as alanine and methionine as well as residues that are too small to exert the same effect as glutamine such as asparagine, the deuterium isotope effect was significantly reduced from the wild type value of 3.44. These results parallel the results obtained from the lysine 56 mutants (11), which also exhibited reduced deu-

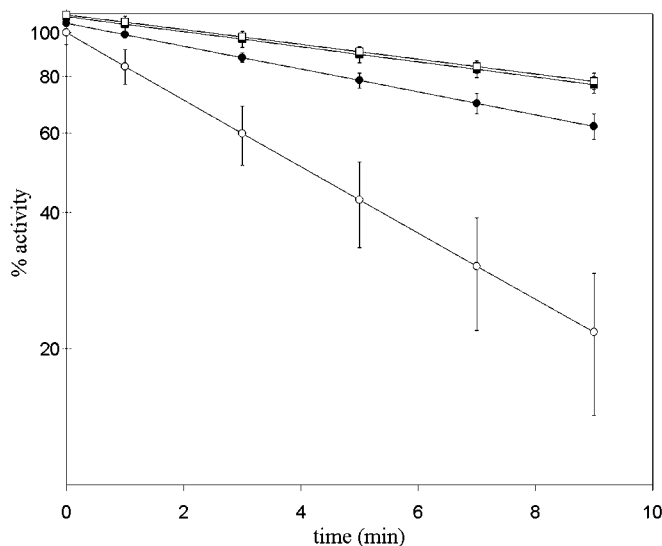


FIG. 2. The effect of diethylpyrocarbonate modification on forward dehydrogenase activity for wild type and mutant DC301 enzymes.  $\circ$ , wild type;  $\blacksquare$ , K56Q;  $\square$ , K56Q/Q100K; and  $\bullet$ , Q100A. Assays were performed using 0.27  $\mu\text{M}$  enzyme and 380  $\mu\text{M}$  DEPC.

terium isotope effects when the lysine was mutated to residues with short side chain length (such as serine and threonine) or was incapable of donating electrons (such as alanine and isoleucine). In such cases, it would appear that a step other than hydride transfer becomes rate-limiting, presumably the activation of the methylene- $H_4$ folate substrate.

In contrast, all mutants at position 100 were completely devoid of  $C_f$  activity. Of particular note is the fact that mutating to residues that either provide the same chemical environment (asparagine) or are of the same size (glutamate) as glutamine produces mutants unable to carry out even residual cyclohydrolase catalysis. These data suggest that glutamine 100 is a critical residue and acts in a very specific manner. Similar results had been obtained with respect to cyclohydrolase catalysis with lysine 56 mutants and formed the basis for the proposal of a catalytic mechanism for DC301 by Schmidt *et al.* (11). However, there are inconsistencies with this mechanism. Their proposed cyclohydrolase mechanism uses an uncharged lysine and generates a protonated species but does not regenerate the starting basic residue for the next cycle. Moreover, although the proposed dehydrogenase mechanism involves lysine 56 to form a covalent adduct with the folate substrate, this residue can be replaced with several others that

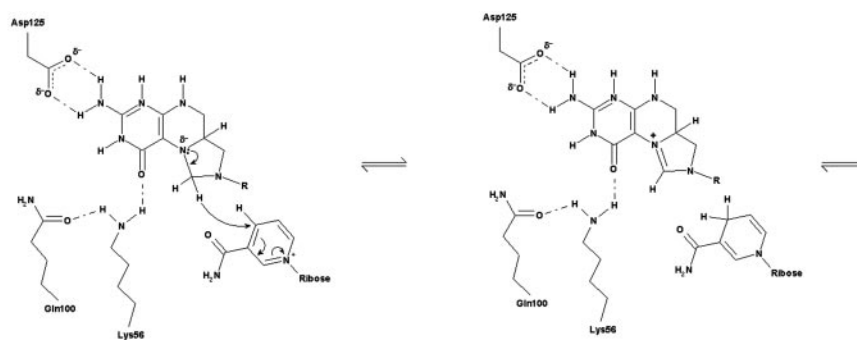
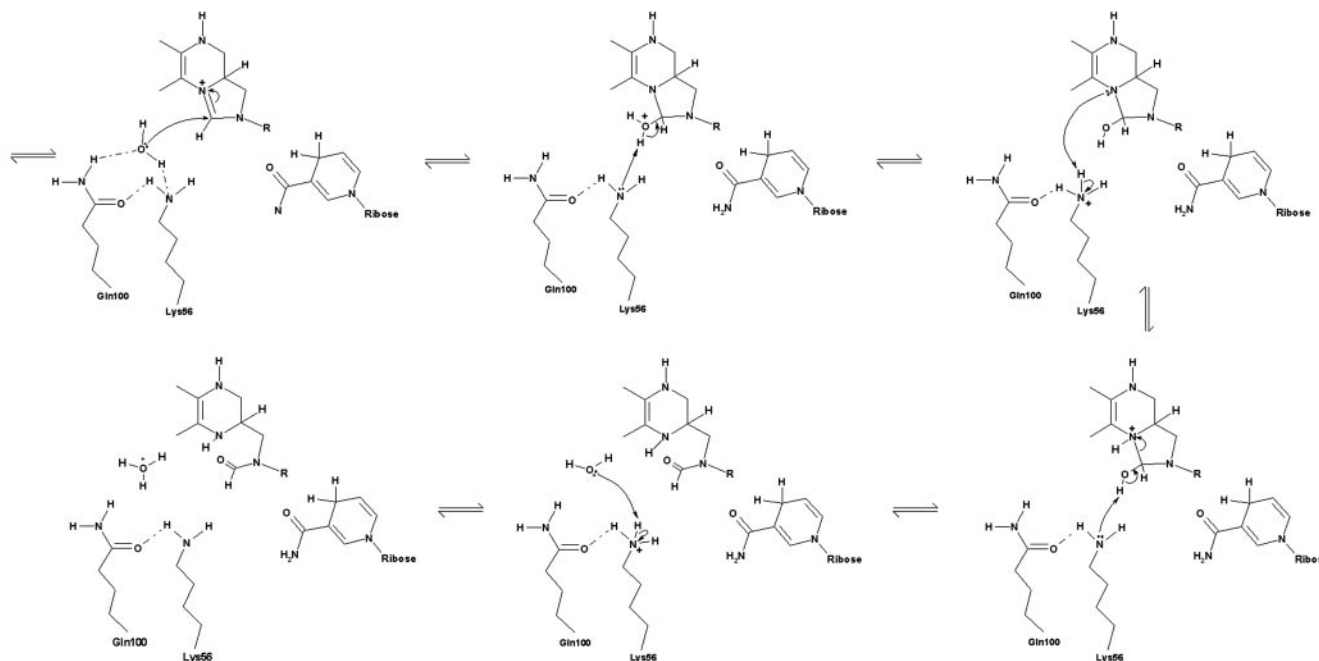
DehydrogenaseCyclohydrolase

FIG. 3. **Proposed catalytic mechanism for forward dehydrogenase and forward cyclohydrolase catalysis in DC301.** 50–60% channeling of the methenyl- $H_4$ folate intermediate is observed in the forward direction, whereas 100% channeling is seen in the reverse direction. Figure was drawn with ISIS Draw 2.3.

are incapable of adduct formation in mutants, which still show significant dehydrogenase activity. The results from this study enable us to describe the mechanism more clearly.

We propose a dehydrogenase mechanism that relies on the general environment provided by lysine 56 and glutamine 100 rather than on any specific interactions with substrates. The results from the K56Q/Q100K double mutant very strongly support this model. Although K56Q or Q100K each retain residual dehydrogenase activity (50 and 8% wild type, respectively), the K56Q/Q100K double mutant restores this activity to near wild type levels (68%). Because the positions of these residues can be effectively interchanged, their effect on catalysis must be a result of the environment that they provide rather than their specific positions with respect to the methylene- $H_4$ folate substrate. We propose that this environment activates the methylene- $H_4$ folate by increasing the electron density of the conjugated ring structure of the tetrahydropteridine moiety, thereby facilitating hydride transfer to the  $NADP^+$  residue.

The proposed cyclohydrolase mechanism requires that the  $\epsilon$ -amino group of lysine 56 be present in a non-protonated form, despite the fact that the  $pK_a$  of the lysine side chain is normally 10.54 (20). From the crystal structure, lysine 56 can be seen embedded in a strongly hydrophobic environment where 8 of the 11 surrounding residues are hydrophobic. Four of these

residues are strictly conserved (Val<sup>280</sup>, Leu<sup>283</sup>, Tyr<sup>52</sup>, and Leu<sup>38</sup>), whereas the others are highly conserved (Met<sup>284</sup>, Ile<sup>40</sup>, Ile<sup>53</sup>, and Leu<sup>98</sup>) (11). Whereas this environment certainly favors a decreased  $pK_a$  for the lysine 56 side chain, the results from the DEPC modification experiments lend more direct functional support for this interpretation.

Smith and MacKenzie (5) succeeded in coincidentally inactivating both activities in the porcine trifunctional enzyme by chemical modification with DEPC. The inactivation was partially protected by NADP and folate. The rate of inactivation was very rapid, indicating that the catalytic residue was probably a histidine. Subsequently, however, the crystal structure did not reveal any histidine residues within the active site. Therefore, we hypothesized that this basic residue may in fact be lysine 56. DEPC can potentially react with several nucleophilic residues. The conditions are chosen to make it a selective reagent that reacts preferentially with available histidine residues both inside and outside the catalytic site. However, DEPC has also been found to react with lysine residues although at a reduced rate from histidine (21, 22). We show that the K56Q and K56Q/Q100K mutants exhibit a 5-fold reduction in the rate of DEPC inactivation, suggesting that lysine 56 is the main DEPC-reactive residue in the enzyme.  $NADP^+$  and folate do not protect against the inactivation of the K56Q mutant, indicating that the residual inactivation is nonspecific.

In order for lysine 56 to react with DEPC at a similar rate to the reaction of DEPC with histidine, the  $pK_a$  of the lysine side chain would have to be reduced from its normal value. Although glutamine does not react with DEPC, a 3-fold decrease in inhibition was seen with the Q100A mutant, which points to the fact that glutamine 100 is a critical residue influencing the reactivity of lysine 56. From the crystal structure, glutamine 100 is seen making a hydrogen bond through its carbonyl group with the  $\epsilon$ -amino group of lysine 56. This interaction is probably the main factor decreasing the  $pK_a$  of lysine 56, because when the interaction is lost in the Q100A mutant, lysine 56 is less reactive to DEPC and cannot fulfill cyclohydrolase catalysis. A similar finding was made in RTEM  $\beta$ -lactamase. From the crystal structure of the *E. coli* enzyme, a catalytic mechanism was deduced, which implies that the  $pK_a$  of the lysine 73 side chain would have to be reduced for catalysis because its proposed role was also to act as a general base. The structure shows the lysine hydrogen bonded to asparagine 132 through the carbonyl group (23) in a manner parallel to the human DC301. Furthermore, when asparagine 132 was mutated to alanine or serine in *Streptomyces albus*, the  $k_{cat}/K_m$  values of the resulting mutants were reduced to 0.5% of the wild type enzyme (24).

The findings from this study including those for aspartate 125 (discussed below) have allowed us to propose a revised catalytic mechanism for the dehydrogenase and cyclohydrolase activities for the bifunctional dehydrogenase-cyclohydrolase (Fig. 3). Although NADPH does not participate in the cyclohydrolase reaction, it has been included for consistency with the previously determined kinetic mechanism (7).

*The Role of Aspartate 125*—Site-directed mutagenesis has shown that the carboxyl group of the conserved Asp<sup>125</sup> is critical for enzyme activity, because the mutation to any other residue without a carboxyl group (including asparagine) completely abolishes both dehydrogenase and cyclohydrolase activities. Indeed, the crystal structure solved with bound Ly345899 inhibitor suggests that Asp<sup>125</sup> makes a hydrogen bond contact through its carboxyl group with the C<sub>2</sub>-amino group of the inhibitor and presumably of the folate substrates. Aspartate residues have been identified as important binding and/or catalytic residues in several folate-dependent enzymes including both human and *E. coli* glycinamide ribonucleotide transformylases (25, 26). In the case of the bacterial glycinamide ribonucleotide transformylase, aspartate 144 is positioned near N<sub>10</sub> of the 10-formyl-H<sub>4</sub>folate substrate and could be involved in binding as well as catalysis (27). Aspartate 120 in *E. coli* methylenetetrahydrofolate reductase serves a catalytic role in folate reduction (28), whereas aspartate 221 in *Lactobacillus casei* thymidylate synthase is involved in folate binding and probably also plays a role in catalysis (29). Because the location of the aspartate contact with the inhibitor in the human DC301 is quite distant from either N<sub>5</sub> or N<sub>10</sub>, the role of this residue would appear to be more significant in substrate binding and positioning than in catalysis.

In addition, when aspartate 125 is mutated to glutamate, the mutation affects the cyclohydrolase (5% residual activity) significantly more than the dehydrogenase (90% residual activity). Because increasing the length of the aspartate by one methylene group in D125E means moving the substrate out of position by that same length, it can be concluded that the cyclohydrolase activity is more dependent on substrate positioning than is the dehydrogenase. This sensitivity of C<sub>f</sub> to substrate positioning may provide an explanation for the differences in substrate channeling observed in each direction.

Although DC301 completely channels the methylenetetrahydrofolate intermediate in the reverse direction, the efficiency is

only 50–60% in the forward direction even though in each direction the second reaction has a greater turnover rate than the first (1). Schmidt *et al.* (11) proposed that channeling would involve a 180° flip of the intermediate to position it for the two reactions, and that this reorientation allows for partial dissociation from the cleft. In addition to the demands of rotating such a large substrate within the cleft, it is not clear how this model would account for the different channeling efficiencies observed in the two directions. Pawelek *et al.* (10) proposed a kinetic description where the cleft includes two partially overlapping subsites with different affinities for the intermediate. In this model, the methenyl-H<sub>4</sub>folate intermediate produced by the C<sub>r</sub> on the side of the cleft with the YXXXX and Ser<sup>49</sup>-Gln<sup>100</sup>-Pro<sup>102</sup> motifs would translocate rapidly to the other side of the cleft near the NADPH residue for reduction to methylene-H<sub>4</sub>folate by the D<sub>r</sub> activity. If translocation in the reverse direction were more rapid than in the forward direction, it would compete more favorably with the rate of dissociation of the intermediate.

However, this model has to be rationalized with all of the studies to date that support the concept of a single, shared bifunctional cleft. Our mutagenesis studies support the notion that the “translocation” can be explained by a stringent requirement for orientation of the intermediate in cyclohydrolase catalysis and a more tolerant one in dehydrogenase catalysis, given the relatively mobile nicotinamide ring. Thus, in the reverse direction, the methenyl-H<sub>4</sub>folate produced by C<sub>r</sub> in the site is efficiently reduced to methylene-H<sub>4</sub>folate. However, in the forward direction, the methenyl-H<sub>4</sub>folate produced by the dehydrogenase is not optimally positioned for cyclohydrolase catalysis, allowing dissociation from the site to compete with repositioning in order to allow hydrolysis. Therefore, the actual translocation is probably a small reorientation of the methenyl-H<sub>4</sub>folate intermediate.

This study has probed the function of several residues in the human bifunctional dehydrogenase-cyclohydrolase domain. In addition to further outlining the role of lysine 56, the roles of glutamine 100 and aspartate 125 have also been defined and related to the mechanisms of catalysis and channeling in DC301.

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