On the Role of Conserved Histidine 106 in 10-Formyltetrahydrofolate Dehydrogenase Catalysis

CONNECTION BETWEEN HYDROLASE AND DEHYDROGENASE MECHANISMS*

The enzyme, 10-formyltetrahydrofolate dehydrogenase (FDH), converts 10-formyltetrahydrofolate (10-formyl-THF) to tetrahydrofolate in an NADP⁺-dependent dehydrogenase reaction or an NADP⁺-independent hydrolase reaction. The hydrolase reaction occurs in a 310-amino acid long amino-terminal domain of FDH (Nt-FDH), whereas the dehydrogenase reaction requires the full-length enzyme. The amino-terminal domain of FDH shares some sequence identity with several other enzymes utilizing 10-formyl-THF as a substrate. These enzymes have two strictly conserved residues, aspartate and histidine, in the putative catalytic center. We have shown recently that the conserved aspartate is involved in FDH catalysis. In the present work we studied the role of the conserved histidine, His106, in FDH function. Site-directed mutagenesis experiments showed that replacement of the histidine with alanine, asparagine, aspartate, glutamate, glutamine, or arginine in Nt-FDH resulted in expression of insoluble proteins. Replacement of the histidine with another positively charged residue, lysine, produced a soluble mutant without hydrolase activity. The insoluble mutants refolded from inclusion bodies adopted a conformation inherent to the wild-type Nt-FDH, but they did not exhibit any hydrolase activity. Substitution of alanine for three non-conserved histidines located close to the conserved one did not reveal any significant changes in the hydrolase activity of Nt-FDH. Expressed full-length FDH with the substitution of lysine for the His106 completely lost both the hydrolase and dehydrogenase activities. Thus, our study showed that His106, besides being an important structural residue, is also directly involved in both the hydrolase and dehydrogenase mechanisms of FDH. Modeling of the putative hydrolase catalytic center/folate-binding site suggested that the catalytic residues, aspartate and histidine, are unlikely to be adjacent to the catalytic cysteine in the aldehyde dehydrogenase catalytic center. We hypothesize that 10-formyl-THF dehydrogenase reaction is not an independent reaction but is a combination of hydrolase and aldehyde dehydrogenase reactions.

* This work was supported by National Institutes of Health Grants DK54388 (to S. A. K.) and DK15289 (to C. W.), by the Medical Research Service of the Department of Veterans Affairs (to C. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The multidomain chimeric enzyme, 10-formyltetrahydrofolate dehydrogenase (FDH), is a natural fusion of two unrelated genes (1). The enzyme converts 10-formyltetrahydrofolate (10-formyl-THF) to tetrahydrofolate (THF) in an NADP⁺-dependent dehydrogenase reaction or an NADP⁺-independent hydrolase reaction (2–4). FDH is a homotetramer of 902-amino acid residue monomers (5, 6). Each monomer consists of two functional domains, amino- and carboxyl-terminal, connected by about a 100-amino acid residue linker which is not part of either catalytic domain (1, 5, 7, 8). The carboxyl-terminal domain of FDH is up to 50% homologous to proteins from a family of aldehyde dehydrogenases (1, 9). Expressed separately, this domain possesses aldehyde dehydrogenase activity (7) as well as the full-length FDH (1). The hydrolase activity resides in a 310-amino acid residue amino-terminal domain of FDH (Nt-FDH) (5, 8) which also can be expressed separately as a functional protein (8). The dehydrogenase reaction requires participation of the second, aldehyde-dehydrogenase homologous, functional domain and occurs only when the two domains work in concert, having been combined in one polypeptide (5, 7, 8).

The amino-terminal domain of FDH bears the folate-binding site and reveals sequence identity to other enzymes that use 10-formyl-THF as a substrate (1, 8). In the cell, 10-formyl-THF is used in three biosynthetic reactions where it serves as a formyl donor (10). Two of the reactions are involved in de novo purine biosynthesis (11) and are accomplished by glycaminide ribonucleotide formyltransferase (GART, EC 2.1.2.2) (12) and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (EC 2.1.2.3) (13). The reactions result in incorporation of two carbons into the purine ring. Another minor biosynthetic reaction that requires 10-formyl-THF is formylation of the amino acid in methionyl-tRNA (14). This is a specialized tRNA used for initiation of translation in prokaryotes, distinct from the one involved in methionine incorporation during polypeptide chain elongation. Such a formylation is also encountered in chloroplasts and mitochondria of eukaryotes (14). The reaction is catalyzed by 10-formyltetrahydrofolate:1-methionyl-tRNA N-formyltransferase (FMT, EC 2.1.2.9) (14).

Despite low overall sequence identity, 10-formyl-THF-utilizing enzymes bear some common structural elements (1, 8). Thus, there are two strictly conserved residues, an aspartate...
and a histidine located within the most conserved region in the middle of the enzyme molecules (1, 8). It has been suggested that these residues participate in folate binding and/or catalysis (1, 15). An aspartate/histidine pair is found as a catalytic dyad or part of catalytic triad in the active site of a number of enzymes including serine proteases (16–18), serine carboxypeptidase (19), phospholipase A_2 (20), dienelactone hydrolase (21, 22), ribonuclease A (23), and a variety of zinc-dependent enzymes (24). From the group of 10-formyl-THF-converting enzymes, the GART mechanism and structure have been studied in more detail (15, 25–27) including resolution of several crystal structures of the enzyme (28–31). It was shown that replacement of the conserved aspartate with asparagine resulted in catalytically inactive enzyme (15). Later it was shown that both conserved residues, the aspartate and the histidine, are adjacent to the folate substrate bound to GART (28, 30) and are involved in the enzyme catalysis (25). Replacement of either of the residues resulted in an enzyme with substantially reduced activity, whereas the double mutant was completely inactive (25). Studies on another enzyme, FMT, also showed that these residues are in close proximity to each other within the substrate binding pocket (32), and they are involved in the enzyme catalysis (33). The third 10-formyl-THF utilizing enzyme, AICAR formyltransferase, that carries out a reaction involved in aminoimidazole carboxamide ribonucleotide formyltransfer function (34). This led to the conclusion that they are just part of a common structural element. Recently, we have shown (35) that similar to GART and FMT, the conserved aspartate and histidine are involved in FDH catalysis. In the present work we studied the role of another conserved residue, histidine 106, in the FDH mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—10-Formyl-5,8-dideoxafolate (10-formyl-DDF) and 5,8-dideoxafolate (DDF) were obtained from Dr. John B. Hynes, Department of Pharmaceutical Chemistry, Medical University of South Carolina. All media were obtained from Difco or Life Technologies, Inc. SDS-PAGE standards were purchased from Amersham Pharmacia Biotech. Other chemicals were obtained from Sigma.

**Site-directed Mutagenesis**—We used the QuikChange site-directed mutagenesis kit (Stratagene) to introduce point mutations into Nt-FDH cDNA cloned to pSin vector (36) or into full-length FDH cDNA cloned to pVL 1393 vector (37). After introduction of mutation the vector was transformed into Escherichia coli XL-1 Blue cells using a standard protocol, and the cells were grown on LB ampicillin plates to allow mutant selection. Plasmids were isolated by using Quantum Prep kit (Bio-Rad) and sequenced to ensure the absence of random mutations.

**Expression of Nt-FDH Mutants**—Expression of Nt-FDH mutants was done as we described earlier (36). The vector with mutation was transformed into E. coli BL21 (DE3) cells (Novagen) according to the manufacturer’s protocol, and the cells were grown in 4 ml of NZCYM medium containing ampicillin (50 mg/ml) overnight at 37 °C with shaking. The 100 ml of NZCYM medium containing ampicillin was inoculated with the overnight culture and incubated at 37 °C with shaking until OD_600 = 0.6 (about 6 h) followed by induction with isopropyl-β-D-thiogalactopyranoside (1 mM final concentration). Three hours after induction the cells were harvested by centrifugation (5,000 × g, 10 min), resuspended in 2 ml of buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 0.1% Triton X-190), and incubated for 30 min with 0.2 mg/ml lysozyme at 37 °C. The suspension was chilled on ice and sonicated (3 times for 30 s each) with the addition of 2% acetone powder. The material (18,000 × g, 15 min). The pellet was dissolved in 5 ml of 0.5 M substrate 10-formyl-DDF. The insoluble material was precipitated by centrifugation (5,000 × g, 10 min), and the soluble sample was examined for the presence of the Nt-FDH by SDS-PAGE and immunoblot technique.

**Purification of Soluble H106K Mutant of Nt-FDH**—The mutant was purified using the procedure that we developed earlier (36) for wild-type Nt-FDH. All buffers used in the purification steps contained 1 mM NaN_3. The supernatant of cell lysate (2 ml) obtained as described above was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.4, and was then passed through a 0.2-mm filter (Nalgene) to remove insoluble contaminants. Purification was done on a DEAE-M-Sephadex A-50 column (1 cm × 20 cm) in a buffer containing 0.1 M NaCl and 10 mM 2-mercaptoethanol. After the dialysis step, the column was equilibrated with 10 mM Tris-HCl buffer, pH 7.4, and fractionated with a linear NaCl gradient (0–0.4 M in 20 mM Tris-HCl, pH 7.4) using a Mono S 1 column (1 cm × 25 cm) in a buffer containing 0.1 M NaCl and 10 mM 2-mercaptoethanol. Nt-FDH was eluted from the affinity column with a linear KCl gradient (0–0.6 M in buffer 1) using a ConSep Exchange Membrane Chromatography cartridge (Millipore) with a linear NaCl gradient (0–0.4 M in 20 mM Tris-HCl, pH 7.4) using a Mono Q column with a linear KCl gradient (0–0.6 M in buffer 1) using a fast protein liquid chromatography system (Amersham Pharmacia Biotech). All buffers used in the purification steps contained 10 mM 2-ME and 1 mM NaN_3. Mutant proteins were purified from the cell-free culture medium by affinity chromatography on a column of Sepharose-5-formyltetrahydrofolate essentially as we described earlier (40). A column (1.5 × 10 cm) was packed with about 8.0 ml of settled gel and equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM 2-ME and 1 mM NaN_3 (buffer 1). Medium (200 ml) plus 2-ME (10 mM) and NaN_3 (1 mM) was applied to the affinity column. The column was then washed with buffer 1 containing 1 mM KCl (100 ml). The enzyme was eluted from the column with buffer 1 containing 1 mM KCl and 20 mM folinic acid. The eluate was passed through a column of Bio-Gel P6-DG (Bio-Rad) equilibrated with buffer 1 to remove unbound folate. The eluate was then passed through a Mono Q column (4 ml × 10 cm) using an Amicon (Beverly, MA) filtration cell. Additional purification was done on a Mono Q column with a linear KCl gradient (0–0.5 M in buffer 1) using a fast protein liquid chromatography system (Amersham Pharmacia Biotech). All assays were performed at 30 °C in a Shimadzu 2401PC double beam spectrophotometer. For measurement of hydrolyase activity, the reaction mixture contained 0.05 mM Tris-HCl, pH 7.8, 100 mM 2-ME, and 5 μM substrate 10-formyl-DDF. 10-Formyl-DDF is an alternative, stable substrate for the enzyme (40). The reaction was started by the addition of 1 μM enzyme (150 μg) in a final volume of 1.0 ml and read against a blank cuvette containing all components except enzyme. Appearance of product 5,8-dideoxafolate (DDF) was measured at 295 nm using a molar extinction coefficient of 18.9 × 10^3 (43). Addition of NADP^+ to the reaction mixture provided a measure of both dehydrogenase and hydrolyase activities, i.e. total activity of the enzyme. Hydrolyase activity measured in the absence of NADP^+ was
subtracted from the total activity to give the dehydrogenase activity. Dehydrogenase activity was also measured independently using the increase in absorbance at 340 nm due to production of NADPH and the molar extinction coefficient of $6.2 \times 10^4$. Aldehyde dehydrogenase activity was assayed using propionaldehyde as described by Lindahl and Evces (44) with our modifications (7). The reaction mixture contained 50 mM CAPS buffer, pH 9.4, 5 mM propionaldehyde, 1 mM NADPH, and enzyme in a total volume of 1 ml. Activity was estimated from the increase in absorbance at 340 nm.

**Fluorescence Studies**—Fluorescence experiments were done on a PerkinElmer Life Sciences 650-40 or Hitachi F-2500 fluorescence spectrophotometer. Emission fluorescence spectra of wild-type and mutant enzymes were recorded by scanning from 300 to 480 nm with fluorescence excitation at either 280 or 295 nm. Protein samples (0.1 mg/ml) were in 20 mM Tris-HCl buffer, pH 7.5. Emission fluorescence spectra of ANS complexes to Nt-FDH were recorded by scanning from 400 to 600 nm with fluorescence excitation at 360 nm. Protein samples (0.1 mg/ml) were preincubated with ANS ($3 \times 10^{-5}$ M) in 20 mM Tris-HCl buffer, pH 7.5, for 30 min. All experiments were done at room temperature.

**Ligand Binding Studies**—Binding of the 10-formyl-DDF and DDF to the Nt-FDH mutants was detected by measuring the quenching of protein tryptophan fluorescence as we described previously (8, 35). Protein samples (about 10.0 nM) were in 50 mM Tris-HCl, pH 7.8. The concentrations of ligands were varied from 2 to 2000 nM for 10-formyl-DDF and from 50 nM to 40 $\mu$M for DDF. The experiments were done at 25 $^\circ$C. Fluorescence excitation was at 291 nm, and the emission was monitored at 340 nm. The data were corrected for dilution effect, for 10-formyl-DDF or DDF intrinsic fluorescence, and for absorptive screening caused by DDF. Intrinsic fluorescence of 10-formyl-DDF or DDF was measured experimentally. In case of DDF the observed fluorescence signal was multiplied by the correction factor, C, to obtained the corrected data. The correction factor was calculated according to the equation $C = 10^{\Delta A/2}$, where $\Delta A$ is the absorbance of DDF at the excitation wavelength (45). $K_{ij}$ values for the ligands were calculated from data on fluorescence quenching in the presence of ligands that were plotted in a linear form. The value ($1 - F_i/F_0$) was plotted against the inverse of ligand concentration (46). This is a modified form of the classical Stern-Volmer plot that relates the decrease in fluorescence to the concentration of a quencher. $F_i$ is intrinsic fluorescence observed at a quencher concentration; $F_0$ is fluorescence in the absence of quencher. The slope of the line (least squares fit) gave a $K_i$ value. Variation of the measured values was about 5%.

**Analysis of Conformation by CD Spectroscopy**—Far-UV CD spectra were obtained on an Jasco 720 spectrometer. The concentration of wild-type and mutant proteins was in the range of 0.3–0.5 mg/ml in 50 mM phosphate, pH 7.0. Spectra were recorded at 25 $^\circ$C using cylindrical quartz cuvette with path length of 0.1 cm. For each sample, three separate spectra were collected and averaged using a step interval of 1.0 nm. The protein spectra were corrected by subtracting a blank solution containing 50 mM phosphate. The final results are reported as mean residue ellipticity in units of degree cm$^2$/dmol$^{-1}$ calculated using a mean residue weight of 115.0.

**Modeling of Structure of Nt-FDH**—Structure of the central region of Nt-FDH molecule excluding 74 amino acid residues was generated using a Swiss-Model server that provides fully automated comparative protein modeling (48–50). Four crystal structures from Brookhaven Protein Data Bank were submitted as templates: 1FMT, 2FMT, 1GAR and 3GAR. The model was visualized and analyzed using Swiss-FDDB Viewer (50) and Rasmol (Glaxo Wellcome).

**RESULTS**

**Site-directed Mutagenesis of Histidine 106 in Nt-FDH**—We mutated histidine 106 in the Nt-FDH sequence to alanine and expressed the mutant in *E. coli*. We found that the mutant was expressed at a high level but as an insoluble protein, although a small portion of it was released into the soluble cell fraction (Fig. 1). We were unable to purify the mutant from the soluble fraction because it precipitated and disappeared on any purification step apparently due to its incorrect folding. This implies that the residue is an important structural element. The fact that the histidine is important to support the protein structure does not mean, however, that the residue is not involved in catalysis also. Therefore, we replaced the histidine with several other amino acids trying to select a suitable substitution that would fit in the protein structure better than alanine. This histidine is strictly conserved in sequences of 10-formyl-THF-utilizing enzymes. Another enzyme, 10-formyl-THF synthetase that synthesizes 10-formyl-THF from THF and formate also reveals elements of the conserved motif (Fig. 2). Besides this motif and the conserved aspartate, there is no further identity between 10-formyl-THF synthetase and 10-formyl-THF-utilizing enzymes. In contrast to 10-formyl-THF-utilizing enzymes, 10-formyl-THF synthetase has glutamate or glutamine instead of the conserved histidine, although the rest of the conserved motif is identical among the proteins (Fig. 2). Therefore, we used these substitutions in order to study whether they would result in expression of soluble protein. We also changed the histidine to the positively charged amino acids, arginine and lysine, and to asparagine which is often used as a replacement for histidine in site-directed mutagenesis. When the mutants were prepared we found that we accidentally introduced aspartate for histidine in another mutant. This mutant was also expressed and studied. Thus, six mutants were expressed as follows: H106N, H106D, H106Q, H106E, H106R, and H106K. Out of the six mutants only H106K was expressed as a soluble protein, although a significant portion (about 40%) was still insoluble (Fig. 1). All other mutants were completely insoluble. No hydrolyase activity was detected in the soluble fraction of the cell lysate for the H106K mutant. To study further the properties of H106K mutant, it was purified using ion-exchange chromatography as we described previously (36). It demonstrated chromatographic behavior during the purification similar to that of the wild-type Nt-FDH (data not shown) and was purified to near-homogeneity (Fig. 3). Analysis of the amino-terminal sequence of the mutant showed that the sequence corresponded to the original sequence of Nt-FDH. The CD and fluorescence spectra of the H106K mutant were identical to those for wild-type Nt-FDH (data not shown). Assay of the hydrolyase activity of the purified H106K mutant showed that it completely lost activity.

**Refolding and Analysis of Histidine 106 Mutants**—We studied whether insoluble His$^{106}$ mutants of Nt-FDH could be refolded into soluble proteins. For this purpose we dissolved the insoluble cell fraction in 6.0 M urea to destroy inclusion bodies and then dialyzed the solution against low salt buffer to remove urea and to recover recombinant proteins. We found that this simple procedure resulted in conversion of a significant portion of insoluble mutants into soluble proteins. About 80–90% of the recombinant proteins was recovered as soluble material. The
Site-directed Mutagenesis of Non-conserved Histidines in the Putative Folate-binding Region—When we studied the role of the conserved Asp$^{142}$ in FDH mechanism, we mutated to alanine all other aspartates located within the putative folate-binding domain. Six non-conserved aspartate residues out of the total of 14 aspartates in Nt-FDH sequence were replaced (35). None of the replacements resulted in change of the enzyme activity, suggesting the importance of Asp$^{142}$. In the present work we used the same approach to ensure that only the conserved histidine is important for catalysis, and loss of the activity was specific due to replacement of a catalytic residue but not due to a general tendency of the enzyme to lose activity in response to amino acid substitutions. Nt-FDH has total of eight histidine residues, four of which are located in the putative folate-binding domain. Replacement of His$^{100}$ and His$^{125}$ with alanine did not reveal any changes in protein expression or in the enzyme activity in comparison to the wild-type Nt-FDH (data not shown). In contrast, the H113A mutant was expressed as an insoluble protein (data not shown). This mutant, however, was recovered after refolding as a soluble protein, and it displayed hydrolase activity identical to that of the wild-type enzyme (Table I) and displayed a similar $K_m$ (7.3 $\mu$m for the native enzyme and 8.8 $\mu$m for the treated enzyme). Based on these experiments we concluded that the unfolding/refolding procedure was adequate to restore the natural conformation of the protein.

Unfolding/Refolding of Wild-type Nt-FDH—As a control experiment wild-type Nt-FDH was subjected to the same unfolding/refolding procedure to study whether it influences protein conformation and activity. After denaturing by dissolving in urea, the wild-type protein renatured during dialysis, and about 100% of the material was recovered as a soluble protein. The refolded wild-type Nt-FDH displayed the same CD and emission fluorescence spectra as non-treated enzyme (data not shown). Analysis of hydrodase activity of the refolded wild-type Nt-FDH showed that it retained almost 100% activity of non-treated wild-type Nt-FDH (Table I) and displayed a similar $K_m$ for both the native enzyme and for the treated enzyme. Based on these experiments we concluded that the unfolding/refolding procedure was adequate to restore the natural conformation of the protein.

Mutant purified in one step from soluble cell fractions as described under “Experimental Procedures.” The other mutants were purified from inclusion bodies (see “Experimental Procedures”). The emission fluorescence maximum wavelength reflects binding of ANS to hydrophobic sites in proteins (52).

**TABLE I**

| Nt-FDH | Hydrodase activity | $K_m$ (nm) |
|--------|--------------------|-----------|
| Nt-FDH | 91                 | 35        |
| Wild-type | 28                | 750       |
| Wild-type$^a$ | 87               | 700       |
| H106A$^b$ | ND                | 830       |
| H106N$^c$ | ND                | 49        |
| H106Q$^c$ | ND                | 900       |
| H106E | ND                 | 65        |
| H106D | ND                 | 60        |
| H106F | ND                 | 70        |
| H106R | ND                 | 55        |
| H106K | ND                 | 84        |
| H106K$^d$ | ND               | 780       |
| H106K$^e$ | ND               | 39        |

$^a$ Protein was unfolded/refolded by dissolving in 6.0 M urea followed by dialysis.

$^b$ ND, non-detectable.

$^c$ Protein was refolded from inclusion bodies.

**FIG. 2.** Sequence alignment of the putative 10-formyl-THF binding motif. The sequences were taken from the references indicated. FH, *E. coli* 10-formyl-THF hydrolase; C$_1$-SYNT, yeast cytoplasmic trifunctional enzyme C$_1$-synthase carboxyl-terminal domain of which is 10-formyl-THF synthetase; 10-FTHFS, *Clostridium acidurici* 10-formyl-THF synthetase.

**FIG. 3.** SDS-PAGE of purified Nt-FDH mutants. Lane 1, H106K mutant purified in one step from soluble cell fractions as described under “Experimental Procedures.” The other mutants were purified from inclusion bodies (see “Experimental Procedures”). Lane 2, H106A; lane 3, H106N; lane 4, H106Q; lane 5, H106E; lane 6, H106R. About 5 $\mu$g of total protein was loaded per lane.
The folate-binding region was replaced (35). All the mutants as well as wild-type enzyme were soluble proteins. In contrast, replacement of His\textsuperscript{106} with several other residues resulted in expression of a completely insoluble protein suggesting that the residue is essential for folding or stability of the entire amino-terminal domain of FDH. This could be the reason that this residue has been preserved during evolution and is consistent with the suggestion that the residue is an important structural element (34). Proteins expressed in \textit{E. coli} are often insoluble because the polypeptide chain does not readily adopt its native conformation due to absence of appropriate conditions (53). In the case where the wild-type protein is soluble but the mutant is insoluble, it could be that the mutation affects protein stability (54). It has been shown, however, that many amino acids in a protein sequence can be freely replaced with little or no effect on protein stability (55, 56). A kinetic model has proposed that the yield of native protein depends only on the rate of protein synthesis, the rate of folding, and the rate of aggregation (57). Thus, alternatively, it could be that substitution of the conserved histidine in N\textsubscript{t}-FDH sequence does not affect protein stability but decreases significantly the rate of folding shifting the reaction to the side of unfolded protein precipitation and formation of inclusion bodies. The question of insoluble recombinant protein refolding has been widely addressed in the literature, and procedures were developed to obtain soluble protein from inclusion bodies (55, 58). In our experiments we were able to recover all histidine mutants as soluble proteins. The refolded mutants were indistinguishable from the wild-type protein as judged by different conformational criteria. They also bind folate ligands with the same affinity as wild-type N\textsubscript{t}-FDH. Based on the above we concluded that refolded mutants adopted conformation inherent to the wild-type protein. Thus, His\textsuperscript{106} is important for correct protein folding rather than for maintenance of the already folded conformation. None of the refolded mutants, however, possessed hydrolase activity, implying that His\textsuperscript{106} is an important catalytic residue. Together with the studies on the only soluble mutant, H106K, the experiments showed that His\textsuperscript{106} is an important structural residue and at the same time is directly involved in enzyme catalysis.

The fact that the conserved aspartate and histidine are essential residues for several 10-formyl-THF-utilizing enzymes implies that these enzymes might have similar catalytic mech-

| Protein   | Expressed as | $V_{\text{max}}$ (mmol/min/mg) | $K_m$ (µM) |
|-----------|--------------|-------------------------------|------------|
| Wild-type | Soluble      | 91                            | 7.0        |
| H100A     | Soluble      | 82                            | 9.5        |
| H113A     | Insoluble    | 84$^a$                        | 9.9$^a$    |
| H125A     | Soluble      | 96                            | 8.3        |

$^a$ Characteristics of the protein refolded and purified from inclusion bodies are shown.

**Table II**

Characteristics of non-conserved histidine mutants of \textit{Nt}-FDH

$V_{\text{max}}$ (mmol/min/mg) and $K_m$ (µM) values for hydrolase activity are shown.

**DISCUSSION**

Earlier we expressed in \textit{E. coli} wild-type N\textsubscript{t}-FDH and several mutants where the conserved aspartate residue in the putative folate-binding region was replaced (35). All the mutants as well as wild-type enzyme were soluble proteins. In contrast, replacement of His\textsuperscript{106} with several other residues resulted in expression of a completely insoluble protein suggesting that the residue is essential for folding or stability of the entire amino-terminal domain of FDH. This could be the reason that this residue has been preserved during evolution and is consistent with the suggestion that the residue is an important structural element (34). Proteins expressed in \textit{E. coli} are often insoluble because the polypeptide chain does not readily adopt its native conformation due to absence of appropriate conditions (53). In the case where the wild-type protein is soluble but the mutant is insoluble, it could be that the mutation affects protein stability (54). It has been shown, however, that many amino acids in a protein sequence can be freely replaced with little or no effect on protein stability (55, 56). A kinetic model has proposed that the yield of native protein depends only on the rate of protein synthesis, the rate of folding, and the rate of aggregation (57). Thus, alternatively, it could be that substitution of the conserved histidine in N\textsubscript{t}-FDH sequence does not affect protein stability but decreases significantly the rate of folding shifting the reaction to the side of unfolded protein precipitation and formation of inclusion bodies. The question of insoluble recombinant protein refolding has been widely addressed in the literature, and procedures were developed to obtain soluble protein from inclusion bodies (55, 58). In our experiments we were able to recover all histidine mutants as soluble proteins. The refolded mutants were indistinguishable from the wild-type protein as judged by different conformational criteria. They also bind folate ligands with the same affinity as wild-type N\textsubscript{t}-FDH. Based on the above we concluded that refolded mutants adopted conformation inherent to the wild-type protein. Thus, His\textsuperscript{106} is important for correct protein folding rather than for maintenance of the already folded conformation. None of the refolded mutants, however, possessed hydrolase activity, implying that His\textsuperscript{106} is an important catalytic residue. Together with the studies on the only soluble mutant, H106K, the experiments showed that His\textsuperscript{106} is an important structural residue and at the same time is directly involved in enzyme catalysis.

The fact that the conserved aspartate and histidine are essential residues for several 10-formyl-THF-utilizing enzymes implies that these enzymes might have similar catalytic mech-

**FIG. 4. SDS-PAGE of FDH H106K mutant on purification steps.**

The mutant was expressed in insect cells and was purified by affinity chromatography on immobilized 5-formyl-THF followed by fast protein liquid chromatography on Mono Q column. Lane 1, culture medium; lane 2, preparation after affinity chromatography; lane 3, preparation after affinity chromatography and chromatography on Mono Q column. St., protein standards as in Fig. 1. About 20 µg (lane 1) and about 3 µg (lanes 2 and 3) of total protein were loaded per lane.
anisms. At the same time the mechanisms should be distinctive since GART/FMT carry out a transferase reaction, whereas the FDH amino-terminal domain carries out a hydrolase reaction. It was found that a third residue, an asparagine, that is two residues upstream of the histidine, is also important for GART and FMT function (25, 33). The fact that FDH does not have the conserved asparagine is probably a reflection of the difference in the enzyme mechanisms. FDH has isoleucine in this position instead of asparagine and that is not even a conservative substitution (1). An enzyme, 10-formyltetrahydrofolate hydrolase, also catalyzing hydrolysis of 10-formyl-THF to THF and formate, was found in *E. coli* (59, 60). Although the *E. coli* hydrolase carries out the same reaction as the FDH hydrolase reaction, it has a higher sequence identity to GART (61) than to the amino-terminal domain of FDH (8). Besides, similar to GART and FMT, the *E. coli* hydrolase has all three putative catalytic residues, an aspartate, a histidine, and an asparagine (59). Although the role of these residues in the enzyme mechanism has not yet been studied, the difference in sequence between *E. coli* hydrolase and FDH might suggest that the same reaction is carried out by two different mechanisms. Indeed, the role of the conserved aspartate and histidine should be more profound in the FDH mechanism than in the *E. coli* hydrolase mechanism since the residues are critical for both FDH activities, hydrolase and dehydrogenase (35). This also suggests that the two FDH mechanisms, the hydrolase and the dehydrogenase, are coupled, and the dehydrogenase mechanism is not independent from the hydrolase mechanism.

Based on crystal structures of two proteins sharing some sequence similarity with *Nt*-FDH, i.e. GART and FMT, we assumed that the conserved histidine is located in a β-sheet near the end of a β-strand. This apparently explains why replacement of the histidine has a greater impact on protein structure compared with replacement of the conserved aspartate in *Nt*-FDH located in the flexible loop. We must mention, however, that replacement of the conserved histidine in FMT did not result in insoluble mutant protein (33). Comparison of crystal structures of FMT (residues 1–189) and GART showed that they have a similar fold despite low sequence identity (32).

**Fig. 5.** Model of the putative folate-binding site/hydrolase catalytic center of *Nt*-FDH. The model of a 74-amino acid long region was generated by using Swiss-Model comparative homology modeling (48–50) and GART/FMT crystal structures as templates. *A*, sequence alignment of the modeling region is shown. Identity for this region is 50% (63.5% including conservative substitution) between *FDH* and FMT and 29.7% (45.9%) between *FDH* and GART. *B*, ribbon structure of the folate-binding site; conserved Asp142 (red) is located in a loop, and conserved His106 (blue) is located in the β-sheet; *C*, spacefill presentation of the model, top view of the folate-binding cavity; *D*, spacefill presentation of the model, side view of the folate-binding cavity. The model was visualized using Rasmol (Glaxo Welcome).

**Fig. 6.** Hydrogen bond network within *Nt*-FDH folate-binding site. The hydrogen bonds (shown by dashed lines) were calculated from the *Nt*-FDH model analyzed with Swiss PDB Viewer (50). Figure shows the hydrogen bond distances. The distances between the side chain oxygens of the conserved Asp142 and the side chain nitrogens of the conserved His106 are as follows: A–C, 4.28 Å; A–D, 4.81 Å; B–C, 3.76 Å; B–D, 3.65 Å.

We suggest that the corresponding region of *Nt*-FDH should also have a similar structure. By using Swiss-Model, a fully automated comparative protein modeling facility (48–50), and GART/FMT structures as templates, we have built a model of the central part of *Nt*-FDH including 74 amino acid residues (Fig. 5A). This region has the highest identity among 10-formyl-THF-utilizing enzymes and includes the conserved histidine and aspartate residues. In FMT and GART structures, this part of the protein sequence forms a hydrophobic cleft that serves as a folate-binding pocket. Although comparative pro-
Fig. 7. Proposed mechanism for 10-formyl-THF hydrolase reaction. The formyl oxygen atom is hydrogen-bonded to His^{106} that helps to withdraw electrons and activates the formyl carbon atom for nucleophilic attack. The side chain oxygen of Asp^{142} tightly binds a molecule of water and activates it by withdrawing a proton. The reaction proceeds by nucleophilic attack of the hydroxyl ion on the formyl carbon atom, resulting in formation of the tetrahedral transition intermediate (step I). This reaction goes through the mechanism of basic catalysis. Electron rearrangement and transfer of a proton from the molecule of water to the cofactor N^{10} is mediated through Asp^{142}. Thus, Asp^{142} works as a relay system withdrawing and donating a proton. The bond between carbon atom of formyl group and N^{10} of folate can be further cleaved to release formic acid and THF (step II).

The present study provides strong evidence that histidine 106 is an essential residue for both hydrolase and dehydrogenase activities of FDH, suggesting that similar to Asp^{142}, His^{106} is involved in both FDH catalytic centers. Analysis of the folate-binding site model revealed that two catalytic residues in the hydrolase catalytic center, His^{106} and Asp^{142}, are located on the bottom of the cavity that forms the substrate-binding pocket and are not exposed on the protein surface (Fig. 5, C and D). The dehydrogenase reaction requires participation of Cys^{707} (6) which serves as a nucleophile and is located inside the carboxyl-terminal domain about 12 Å from the surface as deduced from the crystal structure of aldehyde dehydrogenase (63). Such a structural arrangement should not sterically allow the cysteine to be within the hydrolase catalytic center and be in close contact with the two hydrolase catalytic residues. This in turn suggests that the cysteine does not work simultaneously and in concert with the histidine and aspartate in the enzyme mechanism, but their actions are rather sequential. Thus, we further propose that the 10-formyl-THF dehydrogenase reaction is not a separate reaction but is a conjugation of two sequential reactions as follows: a hydrolase and an aldehyde dehydrogenase. In this case the 10-formyl-THF dehydrogenase mechanism includes the hydrolase reaction as an essential part, and the hydrolase reaction should always precede the dehydrogenase reaction. It also makes sense with respect to the biological role of the hydrolase reaction; the reaction is not an element of a “futile” cycle if it is working together with 10-formyl-THF synthetase but is an essential part of 10-formyl-THF dehydrogenase mechanism. We propose the following mechanism for the hydrolase reaction (Fig. 7). In this mechanism we assume that the tightly bound molecule of water plays the role of a nucleophile or catalytic base. Such a role for a water molecule activated by an aspartate residue has been postulated for a number of enzymes (64–68). A potential catalytic role of a water molecule ordered by a hydrogen bond with the aspartate is located in a flexible loop, the mobility of which is important for the enzyme mechanism (31). Apparently, this hydrogen bond network is necessary to orient catalytically important residues in the right conformation and to fix the flexible aspartate to allow the reaction to proceed. The model further revealed that the conserved Asp^{142} similar to His^{106} is buried in the protein core. Hydrogen bond donors and acceptors that are buried inside the protein normally form hydrogen bonds within the protein (62). However, analysis of the model showed that one oxygen of Asp^{142} side chain is not involved in hydrogen bonding. We suggest that this oxygen is hydrogen-bonded to a molecule of water that is not seen in the model. This suggestion is based on the crystal structure of GART where one ordered water molecule has been found close to the catalytic center (30). Interestingly, the model gives a probable explanation of our results on site-directed mutagenesis of the three non-conserved histidine residues. Two of the residues, His^{100} and His^{125}, are located on the protein surface aside from the hydrophobic cleft, and replacement of these residues did not reveal any changes in N_{2}-FDH properties. The third histidine, His^{113}, substitution of which resulted in expression of insoluble protein, is located close to the conserved His^{106} and is also buried in the protein core. Apparently, this part of the molecule is important for the folding of the entire structure, and replacement of residues within this region can affect the protein properties.
Mechanism of FDH Catalysis

24037

it is in formic acid. However, this step, similar to the aldehyde dehydrogenase reaction, oxidizes the carbon atom just one level up. In the aldehyde dehydrogenase mechanism the substrate forms a thiohemiacetal intermediate with a cysteine residue (69–71). Apparently, in the case of FDH, a similar intermediate is formed. It becomes obvious, however, that a thiohemiacetal intermediate is unlikely to exist within the hydrolase catalytic center, and transfer of an intermediate of the hydrolase reaction to the aldehyde dehydrogenase catalytic center should occur to allow a thiohemiacetal intermediate formation. Further studies are undertaken to explore the dehydrogenase mechanism.

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