Distinct Conformation-mediated Functions of an Active Site Loop in the Catalytic Reactions of NAD-dependent d-Lactate Dehydrogenase and Formate Dehydrogenase*

Revised for publication, January 26, 2005, and in revised form, February 25, 2005
Published, JBC Papers in Press, February 25, 2005, DOI 10.1074/jbc.M500970200

Takehiko Shinoda‡, Kazuhito Arai‡, Mayu Shigematsu-Iida‡, Yoshiro Ishikura‡, Satoru Tanaka‡, Takeshi Yamada‡, Matthew S. Kimber§, Emil F. Pai¶, Shinya Fushinobu¶, and Hayao Taguchi‡**

From the ‡Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, §Affinium Pharmaceuticals Inc., Toronto, Ontario M5J 1V6, Canada, ¶Departments of Biochemistry and Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada, and **Department of Biotechnology, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan.

The three-dimensional structures of NAD-dependent d-lactate dehydrogenase (d-LDH) and formate dehydrogenase (FDH), which resemble each other, imply that the two enzymes commonly employ certain main chain atoms, which are located on corresponding loop structures in the active sites of the two enzymes, for their respective catalytic functions. These active site loops adopt different conformations in the two enzymes, a difference likely attributable to hydrogen bonds with Asn97 and Glu141, which are located on corresponding loop structures of d-LDH and FDH, respectively. X-ray crystallography at 2.4-Å resolution revealed that replacement of Asn97 with Asp did not markedly change the overall protein structure but markedly perturbed the conformation of the active site loop in Lactobacillus pentosus d-LDH. The Asn97→Asp mutant d-LDH exhibited virtually the same $k_{cat}$ value but about 70-fold higher $K_m$ value for pyruvate than the wild-type enzyme. For Paracoccus sp. 12-A FDH, in contrast, replacement of Glu141 with Gln and Asn induced only 5.5- and 4.3-fold increases in the $K_m$ value, but 110 and 590-fold decreases in the $k_{cat}$ values for formate, respectively. Furthermore, these mutants’ FDHs, particularly the Glu141→Asn enzyme, exhibited markedly enhanced catalytic activity for glyoxylate reduction, indicating that FDH is converted to a 2-hydroxy-acid dehydrogenase on the replacement of Glu141. These results indicate that the active site loops play different roles in the catalytic reactions of d-LDH and FDH, stabilization of substrate binding and promotion of hydrogen transfer, respectively, and that Asn97 and Glu141, which stabilize suitable loop conformations, are essential elements for proper loop functioning.

NAD-dependent d- and l-lactate dehydrogenases (d-LDH and l-LDH, EC 1.1.1.28 and EC 1.1.1.27, respectively) are evolutionarily unrelated enzymes (1–3) but catalyze essentially the same reaction, reduction of pyruvate into lactate concomitantly with the oxidation of NADH into NAD+$^+$, with only the chirality of the lactic acid products differing (4). The two enzymes are both equipped with an imidazole-acid pair, His296–Glu264 (5–8) and His195–Asp168 (4), respectively (the numbering of the amino acid residues of d- and l-LDHs is according to that of Lactobacillus pentosus d-LDH (1) and vertebrate l-LDHs (N-system) (9), respectively), as an acid/base catalyst that transfers H$^+$ between the substrate carbonyl oxygen and the solvent. In the case of l-LDH, which is one of the best studied enzymes with respect to structure-function relationships (4, 10), Arg171 and Arg109 in the substrate-binding site promote suitable substrate binding for catalysis (11) and polarization of the bound substrate molecule (12), respectively (Fig. 1A). On the other hand, the substrate-binding site of d-LDH contains only one Arg residue, Arg235, which possibly fulfills the roles of both Arg109 and Arg171 (7, 8, 13). The three-dimensional structure implies that the role of Arg235 as Arg171 may be supplemented by the main chain amide groups of Gly78 and Val79 on a loop structure in the active site, which act as bidentate hydrogen bond donors to the substrate carboxyl group (13, 14) (Fig. 1B).

d-LDH is a member of the d-2-hydroxy-acid dehydrogenase superfamily together with glycerate (15), phosphoglycerate (16–18), and hydroxyisocaproate dehydrogenases (14, 19) and vancomycin-resistant protein H (VanH) (20). In addition, d-LDH structurally resembles NAD-dependent formate dehydrogenase (FDH, EC 1.2.1.2) (21–23) and l-alanine dehydrogenase (24), despite having less than 20% sequence similarity to these enzymes. Unlike the 2-hydroxy-acid dehydrogenases, FDH acts on a single carbon substrate, formate, and converts it to carbon dioxide without H$^+$ transfer between the solvent and the substrate. Nevertheless, FDH possesses the highly conserved residues His312 and Arg284 (the amino acid numbering is according to that for Pseudomonas sp. 101 FDH (23)) at the positions corresponding to His296 and Arg235, respectively (23) (Fig. 1). Compared with their d-LDH counterparts, these residues play different roles in catalysis, interacting with the carbonyl group of the acid substrate that adopts a different orientation in the active site (22, 23, 25, 26) (Fig. 1C). The three-dimensional structure of Pseudomonas FDH (22) shows that the active site also contains a contribution from the main chain of Ile$^{122}$, the residue that corresponds to Val$^{79}$ of d-LDH. In the case of FDH, however, Ile$^{122}$ orients its carbonyl oxygen toward the bound substrate molecule (Fig. 1C), suggesting that the loop is
involved in the promotion of hydride transfer through polarization of the bound substrate, rather than substrate binding (23).

It is notable that the amide and carboxyl side chains of Asn\(^97\) and Glu\(^{141}\), which are also located at corresponding positions in D-LDH and FDH, form hydrogen bonds with the carbonyl and amide groups of the Val\(^78\) and Gly\(^{123}\) main chains on the active site loops, respectively (13, 22) (Fig. 1, B and C). This observation implies that the nature of the hydrogen bonding interaction offered by the residue at position 97/141 may be crucial in determining the conformation of the active site loop in general and the nature of the backbone group presented to the substrate by residue 78/123 in particular.

In this study, we characterized a mutant \textit{L. pentosus} D-LDH (N97D) (27), in which Asn\(^97\) was replaced with Asp, and also two mutant \textit{Paracoccus} sp. 12-A FDHs (E141N and E141Q), in which Glu\(^{141}\) was replaced with Asn and Gln, in order to evaluate the roles of Asn\(^97\) and Glu\(^{141}\) and also, more indirectly, to study the roles of the active site loops in the functioning of the two enzymes.

### EXPERIMENTAL PROCEDURES

**Amino Acid Replacement**—Table I lists the synthetic oligodeoxynucleotides used for site-directed mutagenesis in this study. The replacement of Asn\(^97\) with Asp in \textit{L. pentosus} DCM1558 (ATCC 5841) D-LDH was performed with a Mutagenex kit (Bio-Rad), according to Kunkel (28), using an oligodeoxynucleotide purchased from Takara Shuzo. \textit{Paracoccus} sp. 12-A FDH mutants (E141N and E141Q) were generated using the QuickChange site-directed mutagenesis kit (Stratagene), with oligodeoxynucleotides from GENESOL Oligos. The DNA fragments were sequenced by the dideoxy chain terminator procedure (29) with a DNA sequencer model 4000L (LI-COR) to prove that only the desired mutation had occurred.

**Enzyme Preparation**—The recombinant D-LDHs (6) and FDHs (27) were produced in \textit{Escherichia coli} MV1184 cells harboring expression plasmids for the corresponding enzyme genes, and purification of the recombinant enzymes was performed essentially according to the procedure described previously (6, 30). The purity of the enzyme preparations was examined by SDS-PAGE according to Laemmli (30).

**Enzyme Assay and Protein Determination**—The enzyme assay for D-LDHs was performed at 30 °C in 100 mM sodium MES buffer (pH 5.5) containing 0.1 mM NADH and various concentrations of 2-ketocids (sodium salts). The activity of FDHs toward formate was assayed at 30 °C in 100 mM sodium phosphate buffer (pH 7.0) containing 1.0 mM NAD\(^+\) and various concentrations of sodium formate. The activity of FDHs toward glyoxylate was assayed at 30 °C in 60 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM NADH and various concentrations of glyoxylate. One unit was defined as the conversion of 1 μmol of substrate per min. Kinetic parameters were calculated from plots of \(v/S\) versus \([S]\). The deuterium derivative of NADH (NADD (nicotinamide-4-\(^2\)H)/NADH) was prepared according to Colowick and Kaplan (32) and that of formic acid was purchased from Sigma, to be used for the determination of primary isotope effects on the pyruvate reduction and formate oxidation by the mutant D-LDH and the wild-type and mutant FDHs, respectively. Protein concentrations were calculated using extinction coefficients at 280 nm of 27,045 and 47,330 M\(^{-1}\) cm\(^{-1}\) for D-LDHs and FDHs, respectively, as determined from the amino acid compositions and molecular weights of \textit{L. pentosus} D-LDH (2) and \textit{Paracoccus} sp. 12-A FDH (27).

**Crystallographic Analysis of N97D D-LDH**—The protein solution for crystallization was prepared by concentration of the purified N97D D-LDH sample with a Centriplus YM-10 and Centricon (Millipore). Crystallization of the enzyme was performed at 25 °C by a slight modification of the procedure used for the wild-type enzyme (13, 31), using the hanging drop vapor diffusion method. A 4-μl drop was made by mixing a reservoir solution, which consisted of 50 mM HEPES buffer (pH 7.5), 0.21 M ammonium sulfate, and 28% (w/v) PEG4000 as precipitants, and a protein solution (8 mg/ml), which was then equilibrated against 1 ml of the reservoir solution. Crystals were transferred a solution containing 25% glycerol prior to flash-cooling for data collection.

X-ray data were collected at 100 K with a CCD camera at the BL6A station of the Photon Factory, High Energy Accelerator Research Organization (KEK). Diffraction images were indexed, integrated, and scaled using the DPS/MOSFILM program suite (33, 34). The protein crystals proved to be of space group P1 with unit cell parameters of \(a = 55.7\ \text{Å}, b = 58.9\ \text{Å}, c = 62.3\ \text{Å}, \alpha = 74.5^\circ, \beta = 93.0^\circ, \gamma = 82.5^\circ\), with two molecules per asymmetric unit.

The structure was solved by taking the original protein atom coordinates for the \textit{L. pentosus} D-LDH (13) and subjecting them to rigid...
Functional Main Chain in \(d\)-Lactate and Formate Dehydrogenase

body refinement in Refmac5 (35) from the CCP4 suite, with each catalytic and NADH binding domain defined as a separate rigid body. All subsequent refinements were performed using Refmac5, with 5% of the reflections being randomly excluded from the refinement, and used to monitor \(R_{	ext{free}}\). A maximum likelihood target was used in the refinement protocol, with TLS parameters being used to describe each domain. Following rigid body refinement, the model was subjected to restrained refinement and then rebuilt. Model rebuilding was performed in XtalView (37). To minimize bias, residues 78–82 of each monomer were built into omit \(F_o - F_c\) density (Fig. 2). Val78 from the original model was modified to an aspartate to correct an error in the original published sequence (1). The final model contains 58 water molecules and has an \(R_{	ext{cryst}}\) of 0.224 and an \(R_{	ext{free}}\) of 0.267 (Table II).

**NADH Binding to \(d\)-LDHs**—The dissociation constants (\(K_D\)) of \(N97D\) \(d\)-LDH and NADH were determined essentially according to a previous report (38). The binding of NADH to the \(d\)-LDHs was followed as the change in NADH fluorescence intensity (\(\Delta F\)) essentially according to the method used for \(L\)-LDHs (39), with excitation and emission wavelength of 340 and 460 nm, respectively, using a Jasco FP-750 spectrophotometer. \(\Delta F\) was determined by comparing the fluorescence intensities of NADH in the presence and absence of the enzymes (15 \(\mu M\)) at 30 °C in 50 mM sodium MES buffer (pH 6.0). The \(K_D\) values for the enzymes with NADH were calculated according to the procedure for \(L\)-LDHs (38, 39) by curve fitting with Kaleidagraph.

**RESULTS AND DISCUSSION**

**Effects of Asn97 Replacement on the Conformation of \(L\). pentosus \(d\)-LDH—\(L\). pentosus \(d\)-LDH is a homodimeric protein, with each monomer comprising two domains, a catalytic domain (amino acid positions 1–100 and 300–332) and a coenzyme binding domain (positions 101–298) (13) (Fig. 2A). Asn97 is located on the \(\beta\)-5-\(\alpha\) loop (positions 97–102), which joins the two domains, and Val78 and Gly79 are located on the \(\beta\)-4-\(\alpha\) loop (residues 78–84, called the active site loop in this paper), within the catalytic cleft of the enzyme. The hydrogen bond between Asn57 and Val78 and the conformation of the active site loop in the \(d\)-LDHs (39) is located on the part of the loop (positions 78–82), whereas the dihedral peptide angles for its two subunits of the wild-type enzyme dimer are virtually identical (Fig. 3C). These observations indicate that the Asn97 to Asp replacement destabilizes interactions mediated by the active site loop to the point where there is no longer a well defined, uniquely preferred conformation. It is likely that intermolecular crystal packing interactions help stabilize the two active site loops of the \(N97D\) enzyme dimer in the different conformations in the crystal.

In the case of subunit A, there are particularly marked \(\Delta\phi\) and \(\Delta\psi\) values at the peptide bond between Val78 and Gly79 (Fig. 2D), and consequently the wild-type and \(N97D\) proteins differ greatly in the orientations of the Val78 and Gly79 main chains (Fig. 3C). The hydrogen bond between Val78 and Tyr101 is missing, and the side chain of Tyr101 is disordered in the electron density map in the \(N97D\) enzyme (Fig. 3A). This conformation of the active site loop in the \(N97D\) enzyme appears to be dependent on the flexible nature of Gly79, which can employ its dihedral angles (\(\phi = 124^\circ\), \(\psi = 135^\circ\)) without steric hindrance. In the case of subunit B, on the other hand, there are marked \(\Delta\phi\) and \(\Delta\psi\) values at the peptide bond between Val50 and Asp81, instead of Val78 and Gly79, and the \(N97D\) enzyme preserves the hydrogen bond between the Val78 main chain and the Tyr101 side chain, which is clearly visible in the electron density map (Fig. 3B).

The structures of \(Lactobacillus\) \(bulgaricus\) \(d\)-LDH (40) and \(L\). \(casei\) \(d\)-HicDH (14) have been determined as a binary complex (with NADH) and an abortive ternary complex (with \(\text{NAD}^+\) and 2-ketosicicaprate), respectively. In the binary complex structure of the \(L\). \(bulgaricus\) enzyme, it is notable that the hydroxyl group of Tyr101 (Tyr102 in this enzyme) is shifted from the Val78 (Val79) main chain amide to the nicotinamide ring of NADH. Furthermore, in the ternary complex of \(L\). \(casei\) \(d\)-HicDH (Fig. 2C), Val78 (Val77 in this enzyme) actually forms a hydrogen bond with the substrate carbonyl group, whereas Gly78 (Gly78) does so with the precipitant sulfate ion. In monomer B of the \(N97D\) enzyme, Tyr101 appears to fix the Val78 main chain in a similar orientation of that the wild-type enzyme through the hydrogen bond (Fig. 3). The reorientation of Tyr101 upon ligand binding would result in a loss of the hydrogen bond to the Val78 amide, so substrate binding potentially precipitates changes in the conformation of the active site loop.

The two subunits of the \(N97D\) enzyme also differ both from each other and the wild-type protein in the hydrogen bond network mediated by the active site loop. In the wild-type enzyme, the side chain of Asn82 forms hydrogen bonds with the amide and carbonyl groups of the Asp81 and Lys55 main chains, respectively. In contrast, these two hydrogen bonds are consistently missing in both the two subunits of the \(N97D\) enzyme in which the main chain carbonyl oxygen of Asn82 instead forms a hydrogen bond with the Tyr57 main chain amide. These changes may affect the backbone conformation at positions 54 and 55, which also exhibited significant \(\Delta\phi\) and \(\Delta\psi\) values (Fig. 2D). In the case of the \(N97D\) enzyme, the carbonyl oxygen of Val100 in addition forms a hydrogen bond with the main chain amides of Leu83 and Asn82 in subunits A and B, respectively, whereas these hydrogen bonds are missing in the wild-type enzyme. These new hydrogen bond networks appear to stabilize the two active site loops of the \(N97D\) enzyme dimer in their own conformations in the crystal.

The Asn97 → Asp mutation appears to result in disruption of the structure of the active site loop conformation that is an otherwise conserved feature of all known \(d\)-2-hydroxy-acid dehydrogenase structures. The elevated temperature factors displayed in this region, as well as the adoption of significantly different conformations between the two monomers, indicate that the loop conformation has become sensitive to small per-
Functional Main Chain in d-Lactate and Formate Dehydrogenase

TABLE II
Data collection and model refinement statistics

| Data collection | Values |
|-----------------|--------|
| Resolution range (Å) | 27.5–2.4 |
| Space group | P1 |
| No. observed reflections | 68,843 |
| No. unique reflections | 27,893 |
| Completeness (%) | 94.7 |
| R cryst (%) | 9.4 |
| I/σI | 4.2 |

Refinement

| Values |
|--------|
| R cryst (%) | 22.4 |
| R free (%) | 26.7 |
| Root mean square bonds (Å) | 0.016 |
| Root mean square angles (°) | 1.55 |
| Mean B value | 34.12 |
| No. all atoms* | 5,276 |
| No. water atoms* | 52 |

* Numbers are per asymmetric unit.

Effects of Asn97 to Asp Replacement on the NADH Binding of L. pentosus d-LDH—The Asn97 to Asp replacement potentially affects the NADH binding by d-LDH, as it perturbs Val128, which in the L. casei d-HicDH ternary complex is in van der Waals contact with the nicotinamide moiety of NADH, but also because it appears to weaken the hydrogen bond between Val78 and Tyr123, an interaction that competes with the alternative hydrogen bond between Tyr123 and NADH formed in the ternary complex. As in the case of the wild-type enzyme (38), the fluorescence intensity of NADH markedly increased when NADH was bound to the N97D enzyme (Fig. 4). The titration curves for the wild-type and N97D enzymes gave apparent dissociation constants (K_D) of 2.7 and 0.6 μM for NADH, respectively, indicating that the Asn97 to Asp replacement does not disrupt binding but rather increases the affinity of d-LDH for NADH. The N97D enzyme exhibited a somewhat smaller maximal ΔF than the wild-type enzyme, suggesting that the Asn97 to Asp replacement somewhat changes the environment surrounding the nicotinamide moiety of NADH in the enzyme.

Effects of the Asn97 to Asp Replacement on the Catalytic Activity of L. pentosus d-LDH—Table III summarizes the kinetic parameters of the wild-type and N97D L. pentosus d-LDHs for glyoxylate, pyruvate, 2-ketobutyrate, and hydroxy-pyruvate. In the context of the reduction of pyruvate (the most favorable substrate for the enzyme), the Asn97 to Asp mutant displays a 2 order of magnitude reduction in the catalytic efficiency (k_cat/K_M), but only a relatively minor change in the k_cat value. This contrasts with the Arg235 to Glu (R235Q) and Arg235 to Lys (R235K) mutants, where both the k_cat and k_cat/K_M values for the same substrate are greatly compromised (11). The deuterium derivative of NADH (NADD) gave 1.5-fold lower k_cat and k_cat/K_M for the reaction by the N97D enzyme. This value of the primary isotope effect is in good agreement with the reported value of 1.6 for the wild-type enzyme (11), but much smaller than 2.6 and 2.5 for the R235K or R235Q mutant enzymes, respectively (11), suggesting that the Asn97 to Asp mutant, unlike the Arg235 mutants, is not greatly compromised in its ability to catalyze the hydrate transfer step in the reaction cycle.
The changes in the activation energies, $\Delta G^\ddagger$ and $\Delta G^\ddagger'$, for the Asn to Asp replacement can be calculated from $k_{\text{cat}}/K_M$ ($\Delta G^\ddagger = -RT \ln(k_{\text{cat}}/K_M$ of the mutant enzyme)/$k_{\text{cat}}/K_M$ of the wild-type enzyme)) and $k_{\text{cat}}$ ($\Delta G^\ddagger' = -RT \ln(k_{\text{cat}}$ of the mutant enzyme)/$k_{\text{cat}}$ of the wild-type enzyme)), and were about 2.7 and 0.1 kcal/mol, respectively. These results clearly indicate that Asn97 and, by inference, the active site loop are mostly involved in the stabilization of the enzyme-substrate complexes in both the ground and transition states, playing a role analogous to the Arg$^{171}$ guanidino group in L-LDH (11). The $\Delta G^\ddagger$ of 2.7 kcal/mol for the Asn97Asp mutant is, however, noticeably smaller than the reported $\Delta G^\ddagger$ for the Arg$^{171}$Lys mutant in L-LDH (6 kcal/mol) (11). This likely reflects that the positively charged guanidino group of arginine in L-LDH mediates a stronger interaction with the negatively charged carboxyl group of the substrate than the electroneutral peptide amide groups are capable of in D-LDH (41). In the case of D-LDH, the role of the guanidine group of Arg$^{235}$ in anchoring the substrate carboxyl group (7, 8, 13) likely complements the role of the active site loop in substrate binding, thereby helping better mimic the role Arg$^{171}$ plays in L-LDH.

![FIG. 3. Structure of the active site loop in the N97D L. pentosus d-LDH.](image)

A, $2(F_o - F_c)$ electron density map contoured at 1σ for the vicinity of Asp97 in subunit A of the N97D d-LDH. This figure was made with XtalView (37) and rendered with Raster3D (50). B, similar to A, except showing the analogous region of subunit B. C, stereo diagram showing a superposition of the catalytic domain in the region of residue 97 for the wild-type L. pentosus d-LDH subunit A (gray), N97D subunit A (cyan) and Asn97 subunit B (green).

**TABLE III**

| Substrate   | $K_M$ (mM) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_M$ (s$^{-1}$/mM) |
|-------------|------------|-----------------------------|----------------------------------|
| Wild-type   |            |                             |                                  |
| Pyruvate    | 0.12       | 320                         | 2,700                            |
| Glyoxylate  | 5.4        | 270                         | 49                               |
| 2-Ketobutylate | 8.1       | 120                         | 15                               |
| Hydroxypyruvate | 0.28     | 260                         | 920                              |
| Oxamate ($K_f$) | 2.8        |                             |                                  |
| Formate ($K_f$) | 44         |                             |                                  |
| N97D        |            |                             |                                  |
| Pyruvate    | 8.2 (69)   | 270 (0.84)                  | 33 (1.2 × 10$^{-2}$)             |
| Glyoxylate  | 72 (13)    | 50 (0.19)                   | 0.7 (1.4 × 10$^{-2}$)            |
| 2-Ketobutylate | 58 (7.2)  | 4.5 (0.04)                  | 0.08 (5.3 × 10$^{-3}$)           |
| Hydroxypyruvate | 29 (104) | 38 (0.15)                   | 1.3 (1.4 × 10$^{-2}$)            |
| Oxamate ($K_f$) | 43 (15)   |                             |                                  |
| Formate ($K_f$) | 140 (3.2) |                             |                                  |

The changes in the activation energies, $\Delta G^\ddagger$ and $\Delta G^\ddagger'$, for the Asn to Asp replacement can be calculated from $k_{\text{cat}}/K_M$ ($\Delta G^\ddagger = -RT \ln(k_{\text{cat}}/K_M$ of the mutant enzyme)/$k_{\text{cat}}/K_M$ of the wild-type enzyme)) and $k_{\text{cat}}$ ($\Delta G^\ddagger' = -RT \ln(k_{\text{cat}}$ of the mutant enzyme)/$k_{\text{cat}}$ of the wild-type enzyme)), and were about 2.7 and 0.1 kcal/mol, respectively. These results clearly indicate that Asn97 and, by inference, the active site loop are mostly involved in the stabilization of the enzyme-substrate complexes in both the ground and transition states, playing a role analogous to the Arg$^{171}$ guanidino group in L-LDH (11). The $\Delta G^\ddagger$ of 2.7 kcal/mol for the Asn97Asp mutant is, however, noticeably smaller than the reported $\Delta G^\ddagger$ for the Arg$^{171}$Lys mutant in L-LDH (6 kcal/mol) (11). This likely reflects that the positively charged guanidino group of arginine in L-LDH mediates a stronger interaction with the negatively charged carboxyl group of the substrate than the electroneutral peptide amide groups are capable of in D-LDH (41). In the case of D-LDH, the role of the guanidine group of Arg$^{235}$ in anchoring the substrate carboxyl group (7, 8, 13) likely complements the role of the active site loop in substrate binding, thereby helping better mimic the role Arg$^{171}$ plays in L-LDH.
In contrast with the case for pyruvate, the $k_{cat}$ values for alternative substrates were markedly reduced by the Asn$^{97}$ replacement (Table III). Although the wild-type enzyme exhibited comparable $k_{cat}$ values for pyruvate and the alternative substrates, the N97D enzyme exhibited much lower $k_{cat}$ values for these substrates. Such a kinetic phenomenon, which also occurs in the cases of the Arg$^{171}$ replacement in L-LDH (11) and Arg$^{235}$ replacement in D-LDH (7), suggests that the N97D enzyme tends to form an inappropriate or nonproductive enzyme-substrate complex for alternative substrates, which gives smaller apparent $k_{cat}$ and $K_M$ values than the real values in the kinetics (42), as in the case of the Arg$^{171}$ mutant L-LDH (11). It is plausible that the N97D enzyme tends to bind carboxy acid substrates in an orientation similar to that in which FDH binds formate because, like FDH, the enzyme lacks the interactions between the main chain amides and the substrate carboxyl group (Fig. 1). D-LDH possesses a pocket that includes Tyr$^{32}$ that binds the C-3 methyl group of pyruvate (38). This binding pocket may be the key to preferentially stabilizing the authentic substrate, pyruvate, in a productive binding mode in the active site but fails to do so for alternative substrates where a different substituent is present on C-2.

The N97D enzyme exhibited a 15-fold increased inhibition constant ($K_I$) for oxamate, an inert pyruvate analogue, as compared with the wild-type enzyme (Table III). On the other hand, formate also inhibited the reaction of the enzyme of the substrate pyruvate in a competitive manner, and the N97D enzyme exhibited significantly, but only slightly, increased formate $K_I$, suggesting that formate is more apt to be bound to the enzyme in the “incorrect” i.e. “FDH-like” orientation, which is less dependent on the active site loop. This explanation is plausible as formate lacks the 2-carboxyl group that promotes the “correct” orientation of substrate binding through an interaction with Arg$^{235}$ and NADH.

**Effects of Replacements of Glu$^{141}$ on the Catalytic Activity of FDH toward Formate**—Based on the three-dimensional structure of Pseudomonas sp. 101 FDH (22), it has been proposed that the Ile$^{122}$ carbonyl oxygen of the active site loop polarizes the nearby substrate molecule, and thereby stimulates the rate-limiting hydride transfer step (23). Paracoccus sp. 12-A FDH exhibits 83.5% amino acid identity with Pseudomonas sp. 101 FDH (27). We characterized two mutant Paracoccus FDHs, the E141N and E141Q enzymes, which mimic L. pentosus D-LDH in the nature of the Asn$^{97}$ side chain, to evaluate the crucial role of the active site loop in the catalytic function of FDH.

Table IV summarizes the kinetic parameters for formate oxidation by the wild-type and E141N and E141Q mutant Paracoccus sp. 12-A FDHs. The E141N and E141Q enzymes exhibited greatly reduced catalytic efficiency ($k_{cat}/K_M$) as compared with the wild-type enzyme, 3 and 4 orders of magnitude, respectively. For both mutant enzymes, the reduction of $k_{cat}/K_M$ was mainly due to decreases in the $k_{cat}$ values ($10^8$-$10^9$-fold) rather than increases in the $K_M$ values (less than 10-fold). This is in marked contrast to the case of the Asn$^{97}$ to Asp replacement in D-LDH (Table III). When the changes in kinetic parameters were converted to activation free energy changes, the changes with the Glu$^{141}$ to Asn and Glu replacements correspond to $\Delta G^*_D$ of 4.8 and 3.9 kcal/mol and $\Delta G^*$ of 3.9 and 2.8 kcal/mol, respectively.

The deuterium derivative of formate did not significantly affect the $K_M$ value but led to a 2.4-fold reduction in the $k_{cat}$ value for the wild-type Paracoccus enzyme (Table IV). This indicates that the hydride transfer step is rate-limiting in the catalytic reaction of the enzyme, as in the case of most FDHs, which exhibit the deuterium primary isotope effect of 2.1–3.1 (23). Furthermore, the E141N and E141Q enzymes exhibited even larger isotope effects (2.8) than the wild-type enzyme (Table IV). These results indicate that the decrease in $k_{cat}$ by the Glu$^{141}$ replacements is actually due to impairment of the hydride transfer step. Therefore, the presence of Glu$^{141}$, and by inference the Ile$^{122}$ carbonyl oxygen presented by a correctly oriented active site loop, enhances the hydride transfer by at least 2–3 orders of magnitude.

**Effects of Replacements of Glu$^{141}$ on the Catalytic Activity of FDH as to Glyoxylate Reduction**—The reaction catalyzed by FDH does not require any acid/base catalyst such as His$^{296}$ in D-LDH, which transfers H$^+$ between substrate and solvent. Nevertheless, the enzyme is equipped with conserved His$^{322}$ at the position corresponding to His$^{296}$, together with conserved Arg$^{264}$ at the position of Arg$^{235}$ (23) (Fig. 1), using it for a different role (23, 25, 26). Although Glu$^{264}$, which supports the catalytic function of His$^{296}$ in D-LDH, is replaced with conserved Gln (Gln$^{313}$) in FDH, Glu$^{264}$ is not essential for the catalytic function of D-LDH (5, 8), suggesting that His$^{322}$ of FDH should be able to act as an acid/base catalyst in a manner analogous to His$^{296}$ of D-LDH. FDH therefore appears to be equipped with all of the basic catalytic machineries to allow it to act as a 2-hydroxy-acid dehydrogenase, except for the unsuitable conformation of the active site loop. It might be anticipated that the replacement of Glu$^{141}$ could convert the enzyme into a 2-hydroxy-acid dehydrogenase by repositioning the active site loop conformation into something that resembles that seen in D-LDH. We therefore investigated the activity of the mutant FDHs as to 2-ketoacid reduction.

Table V summarizes the catalytic parameters of the wild-type and mutant enzymes for glyoxylate reduction. The wild-type enzyme exhibited low catalytic activity toward glyoxylate, giving the $k_{cat}/K_M$ of 9.3 × 10$^{-4}$ s$^{-1}$, which was 8.5 × 10$^{-4}$-fold lower than the $k_{cat}/K_M$ for formate oxidation (Table IV). In contrast, the E141N and E141Q enzymes exhibited 85 and 9.2-fold higher $k_{cat}/K_M$ for glyoxylate than the wild-type enzyme, and consequently 200 and 5.2-fold higher activity for glyoxylate reduction than those for formate oxidation, respectively. This result indicates that the single amino acid replacement of Glu$^{141}$ drastically converts Paracoccus FDH to a glyoxylate reductase (EC 1.1.1.26), i.e. a 2-hydroxy-acid dehydrogenase, and thus demonstrates that the conformation of the active site loop actually determines the enzyme function between FDH and 2-hydroxy-acid dehydrogenase.

It is also notable that the Glu$^{141}$ to Asn replacement converts the enzyme function much more drastically than the Glu$^{141}$ to Gln replacement, more drastically increasing the activity toward glyoxylate (Table V) and decreasing toward formate (Table IV). Potentially Asn$^{141}$, being less bulky than Gln, shifts the active site loop over slightly and therefore leaves a little more room for the glyoxylate molecule (which requires significantly more room to bind productively than does formate). The more favorable active site for 2-ketoacid reduction exhibited by Asn$^{141}$ FDH is consistent with the fact that most D-LDHs and
related 2-hydroxy-acid dehydrogenases are highly conserved for an Asn residue at the corresponding position (Fig. 5).

It is expected that the increased activity of the E141N enzyme is mainly due to improvement of glyoxylate binding, because the Glu141 replacement possibly introduces new hydrogen bonds with the carboxyl group of a substrate like those of D-LDH forms with pyruvate as well as clearing more space (Fig. 1B). Nevertheless, the E141N enzyme exhibited a less than 2-fold improved $K_M$ value and a 50-fold increased $k_{cat}$ for glyoxylate as compared with the wild-type enzyme (Table V).

This result is not strange if it is assumed that the kinetics of glyoxylate reduction also includes a nonproductive enzyme-glyoxylate complex, which gives smaller apparent $k_{cat}$ and $K_M$ values than the real values (42). It is possible that the glyoxylate molecule may preferentially bind in the same orientation as productively bound formate, particularly in the case of the wild-type enzyme, which lacks the active site loop-substrate carbohydrate group interactions that might assist in correctly orienting the substrate glyoxylate in the active site for reduction. Therefore, in this case only $k_{cat}/K_M$ values should be compared, because these values are insensitive to such a nonproductive complex. The change in $k_{cat}/K_M$ with the Glu to Asn replacement (85-fold difference) corresponds to $\Delta G^\ddagger$ of 2.7 kcal/mol, which is in good agreement with $\Delta G^\ddagger$ (2.7 kcal/mol) for the Asn97 to Asp replacement in D-LDH.

Together with the wild-type enzyme, neither mutant FDH enzymes showed any detectable catalytic activity toward pyruvate (data not shown). Because the active site of FDH is optimal for formate binding, and its size is much smaller than that of D-LDH (22, 23), glyoxylate which, with hydrogen on C-2 is the smallest of the 2-ketoad substrates, appears to be the only 2-ketoad that can be accommodated within the binding site of FDH. On the other hand, the N97D D-LDH showed no detectable catalytic activity with respect to formate oxidation (data not shown). It was not evident on the basis of structural analysis that the active site loop of the N97D enzyme is in a suitable conformation to support formate oxidation (Fig. 3). In addition, D-LDH possesses a larger and less favorable binding site for formate than FDH and lacks some other essential catalytic machinery that in FDH promotes formate oxidation, such as Asn146 and Pro97-Phe86 (23). The conversion of D-LDH to FDH therefore seems not to be readily accomplished only through the minor structural changes induced by a single amino acid replacement.

Conservation of Asn97 in D-LDHs and D-LDH-related 2-Hydroxy-acid Dehydrogenases and Glu141 in FDHs—Although bacterial D-LDHs are highly divergent, even within the Lactobacillus enzymes (43), sequence comparison revealed that Asn97 is highly conserved not only in most bacterial D-LDHs but also related D-2-hydroxy-acid dehydrogenases from various organisms (Fig. 5), suggesting that all these enzymes employ corresponding active site loops for common roles in their catalytic reactions. Nevertheless, the comparison also indicated that Asn is not the only amino acid that can fulfill the role of Asn97, because it is replaced with Arg and Tyr in the cases of E. coli D-LDH (44) and human hydroxypyruvate/glyoxylate reductase (45), respectively. Arg and Tyr have much larger side chains than Asn, although they consistently have the ability to form hydrogen bonds with the carbonyl groups of the peptide main chains.

### Table V

| Enzyme     | $k_{cat}$ (s$^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ | $K_M$ (mM) |
|------------|---------------------|------------|---------------|------------|
| Wild-type  | 7.5                 | 7.0 $\times$ 10$^{-3}$ | 9.3 $\times$ 10$^{-4}$ | 8.5 $\times$ 10$^{-4}$ |
| E141N      | 4.6 (0.61)          | 3.6 $\times$ 10$^{-1}$ (51) | 7.8 $\times$ 10$^{-2}$ (84) | 2.0 $\times$ 10$^2$ (2.4 $\times$ 10$^2$) |
| E141Q      | 2.6 (0.35)          | 2.3 $\times$ 10$^{-2}$ (3.2) | 8.8 $\times$ 10$^{-3}$ (9.5) | 5.2 (6.1 $\times$ 10$^3$) |

* $k_{cat}/K_M$ and $(k_{cat}/K_M)^*$ indicate the $k_{cat}$/$K_M$ values in the glyoxylate reduction and formate oxidation, respectively.
Moritani-Otsuka, C., DNA Data Bank of Japan (DDBJ) accession number AB0050737-1) and Mastigamoeba balmuthi (Moore, D. V., and Muller, M., DDBJ accession number AY313608-1) enzymes because of their low amino acid identities with bacterial enzymes, as well as mammal D-LDHs, which belong to a different enzyme superfamily from bacterial D-LDHs (46). On the other hand, Glu141 is more strictly conserved in known FDHs from various sources such as bacteria, yeast, fungi, and plants (Fig. 5), suggesting that these enzymes commonly employ the active site loop for the same role that is mediated by Glu141.

Conclusions—Although the main chain atoms of a protein are often involved in the protein function directly, it is generally difficult to evaluate the crucial roles of such main chains by conventional approaches of protein engineering. In the cases of D-LDH and FDH, which resemble each other structurally but differ in catalytic function, we could successfully evaluate their functional main chains on the corresponding active site loops by mutating a key residue that orients and positions the loop to the counterpart residue in the corresponding structure.

The results obtained were clearly consistent with the roles of these active site loops that were previously proposed on the basis of structural analysis (13, 14, 23, 22). In the case of D-LDH, the main chain amide groups of the loop seem to stabilize the binding of a substrate in the proper orientation for the catalytic action of D-LDH through interactions with the carboxyl oxygens of the substrate, in analogy to the role played by the side chain of Arg71 in 1-LDH (Fig. 1). The structural conservation of this motif, and the general conservation of Asn97 in most D-LDH-related 2-hydroxy-acid dehydrogenases, argues that this role for the active site loop is a conserved element of the catalytic machinery in this enzyme family. In the case of FDH, on the other hand, the carbonyl groups on the main chains of the loop stimulate the hydride transfer step of catalysis by polarizing the substrate formate and, perhaps less critically, destabilizing binding in the unproductive D-LDH-like binding mode.

In addition, this study clearly indicates that these distinct functions of the active site loop depend upon the distinct conformations stabilized by Asn97 in D-LDH and Glu141 in FDH. It is particularly noteworthy that the Glu141 → Asn substitution converts Paracoccus FDH to reasonably active glyoxylate dehydrogenase, indicating that the replacement at this position is a key in the divergence of FDHs and 2-hydroxy-acid dehydrogenases. The multiplicity in the conformation-mediated functions of the active site loop appears to pivotal in the versatility of the common structural framework in the superfamily of D-LDH and FDH.

REFERENCES

1. Taguchi, H., and Ohta, T. (1991) J. Biol. Chem. 266, 12588–12594
2. Bernard, N., Ferman, T., Garmyn, D., Hols, P., and Delcour, J. (1991) FEBS Lett. 290, 2029–2034
3. Kochhar, S., Hunziker, P. E., Leong-Morgenthaler, P., and Hottinger, H. (1992) J. Biol. Chem. 267, 6885–6890
4. Flick, M. J., and Konieczny, S. F. (2002) Biocatal. Bioangiogenes. 18, 347–355
5. Hoekstra, C., Ishikura, Y., and Chiu, M. T., Motoh, H., Tazuki, S., Nakahara, Y., Tamura, Y., Shinoda, T., and Taguchi, H. (2003) J. Biol. Chem. 278, 5023–5036
6. Fersht, A. R. (1977) Enzyme Structure and Mechanism, pp. 839–846, W. H. Freeman & Co., San Francisco
7. Bernard, N., Johansen, K., Ferman, T., Garmyn, D., Hols, P., Holbrook, J., and Delcour, J. (1994) Eur. J. Biochem. 224, 439–446
8. humorous text about the importance of the main chain in D-LDH and FDH function.