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Involvement of Glu-264 and Arg-235 in the Essential Interaction between the Catalytic Imidazole and Substrate for the D-Lactate Dehydrogenase Catalysis[1]

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For *Lactobacillus pentosus* D-lactate dehydrogenase, the binding of 2-ketoacids is markedly stabilized through interactions between the protonated imidazole of His-296, an acid/base catalyst of the enzyme, and the carbonyl oxygen of 2-ketoacids. The replacement of Arg-235 with Gln destabilized the inhibitory binding of oxamate much more than that of formate, acetate, or propionate, and the Arg to Lys substitution specifically diminished only oxamate binding. On the other hand, replacement of a conserved Glu, Glu-264, with Gln severely impaired the enzyme activity and markedly reduced affinity to 2-keto acids. The pH dependence of the oxamate inhibition revealed that the substitutions of Arg-235 and Glu-264 induced a great loss of the imidazole-carbonyl interaction. However, replacement of Glu-264 with Asp, another acidic amino acid, affected the enzyme function less than the Glu to Gln substitution. In addition, both the Arg-235 and Glu-264 substitutions induced marked increases in the primary isotope effect on the catalysis, suggesting that these amino acids stimulate the hydrogen transfer step in the catalysis. We concluded, therefore, that the guanidino and carboxyl groups of Arg-235 and Glu-264, respectively, cooperatively promote the essential imidazole-substrate interaction, enhancing the substrate binding and catalysis.

Key words: active center, D-lactate dehydrogenase, *Lactobacillus*, stereospecificity.

Many eukaryotic and prokaryotic cells possess an NAD-linked L- or D-lactate dehydrogenase (EC 1.1.1.27 or EC 1.1.1.28, respectively), depending on the species (*1-3*). The two enzymes catalyze virtually the same reaction, except for the chirality of the lactic acid products, and apparently play the same physiological role in the glycolysis pathway, acting at the last step of glycolysis under anaerobic conditions. L- and D-LDHs, however, belong to evolutionally distinct enzyme families, the L- and D-2-hydroxyacid dehydrogenase families, respectively (*4-6*). The D-enzyme family includes a number of proteins with different physiological functions and substrate specificities, and even non-2-hydroxyacid dehydrogenases such as formate dehydrogenase (FDH) (*7*). Recently, the three-dimensional structure of D-LDH of *Lactobacillus pentosus*, previously called *Lactobacillus plantarum*, was reported (*8, 9*), together with those of formate (*10*), D-glycerate (*11*), and D-phosphoglycerate (*12*) dehydrogenases, other members of the D-enzyme family. The protein structures of the D-enzymes resemble one another but differ from those of the L-enzymes.

Biochemical studies on *L. pentosus* D-LDH indicated that His-296 acts as an acid/base catalyst (*13*), and Arg-235 plays an important role in the substrate binding (*14*), like His-195 and Arg-171 of L-LDH (*15*), respectively. Although no ternary complex structure in D-type enzymes has yet been found, except for that of FDH (*10*), the proposed models (*9-12*) are consistent with the results of biochemical studies (*13, 14*). However, less is known about the structure-function relationship of the D-type enzymes than that of the L-type of enzymes (*16, 17*).

In the case of L-LDH, Asp-168, which forms the acid-imidazole pair with His-195, the acid/base catalyst of the enzyme, enhances the catalytic function of His-195 through the stabilization of the enzyme-pyruvate and transition state complexes (*18*), and Arg-109 also plays an essential role in the catalysis through the polarization of the carbonyl group of pyruvate (*19*). The proposed models for the D-enzyme ternary complex (*9-11, 20*) consistently indicate that a conserved Glu, Glu-264, in D-LDH forms hydrogen bonds with the catalytic His imidazole, and is the most possible counterpart of Asp-168. However, replacement of Glu-264 with Gly in *L. bulgaricus* D-LDH induced no marked reduction in the enzyme activity but a great shift in the pH-dependence for the activity, suggesting that Glu-264 only modulates the pH dependence of the enzyme

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Abbreviations: DEPC, diethylpyrocarbonate; LDH, lactate dehydrogenase; FDH, formate dehydrogenase; L-LDH, L-lactate dehydrogenase; Hepes, *N*-2-hydroxyethylpiperadine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

catalysis, unlike Asp-168 of L-LDH (*21*). A counterpart of Arg-109 also remains unclear, and is missing in some of the models (*10, 11*). Using the apo-enzyme structure of *L. pentosus* D-LDH as a guide, however, Stoll *et al.* proposed model in which Arg-235 of the enzyme also fulfils the role of Arg-109 (*9*).

To elucidate the crucial roles of Arg-235 and Glu-264 in *L. pentosus* D-LDH, we characterized mutant enzymes as to His-296, Arg-235, and Glu-264 in detail. In this paper, we report that the guanidino group of Arg-235 and the carboxyl group of Glu-264 greatly support the interaction between His-296 and substrate that is essential for the substrate binding and catalysis.

MATERIALS AND METHODS

Chemicals—The enzymes for DNA manipulation were purchased from Boehringer-Mannheim, Takara Shuzo (Kyoto), and Toyobo Biochemicals (Tokyo). The deuterium derivative of NADH [NADD, (*nicotinamide*-4-^{2}H)NADH] was prepared according to Colowick and Kaplan (*22*), using yeast alcohol dehydrogenase (Boehringer-Mannheim).

Culture Conditions and Enzyme Purification—Cultivation of *Escherichia coli* cells harboring an expression plasmid for the wild-type or mutant *L. pentosus* D-LDH gene, and purification of the enzyme were performed as described previously (*13*), but the cells for two of the mutant enzymes, in which His-296 was replaced with Tyr and Gln (*13*), respectively, were cultivated at 25°C for the enzyme production, because the two enzymes tended to be produced as inclusion bodies, inactive and insoluble forms of the enzymes, particularly when the *E. coli* cells were cultured at above 30°C. The lower temperature for cultivation markedly increased the population of the soluble and active forms of the enzymes and reduced the insoluble form (data not shown). The purity of the enzyme preparations was examined by SDS-PAGE according to Laemmli (*23*).

Enzyme Assay and Protein Determination—The assay for enzyme activity was performed at 30°C, and pyruvate reduction and D-lactate oxidation were measured in assay mixtures containing 0.1 mM NADH and 10 mM NAD^+, respectively. Protein concentrations were determined with the Pierce bicinchoninic acid (BCA) protein reagent (*24*), using bovine serum albumin as a standard protein.

Site-Directed Mutagenesis—Oligodeoxynucleotides, 5′-CCT ACG AAT ACC AAA CTA AGA TCT-3′ and 5′-CCT ACG AAT ACG ATA CTA AGA TCT-3′, were synthesized to replace Glu-264 with Gln and Asp, respectively, using an Applied Biosystems model 391 DNA synthesizer. Site-directed mutagenesis was performed with a MUTA-GENE *in vitro* mutagenesis kit (Bio-Rad), according to Kunkel (*25*), and the DNA fragment was sequenced by the dideoxy chain terminator procedure (*26*) using an Applied Biosystems model 370A DNA sequencer.

RESULTS

Determination of the Exact Catalytic Constant of Gln-296 Mutant Enzyme—To evaluate the crucial involvement of the imidazole-carbonyl interaction in pyruvate binding, a higher amount of the Gln-296 enzyme was prepared according to the improved enzyme production protocol (see "MATERIALS AND METHODS") and used for the enzyme assay (up to 0.2 mg/ml in the assay mixture). The assay gave reproducible k_{cat} and pyruvate K_M values below pH 6.5 (Fig. 1), though the enzyme activity was still too low to obtain reliable values above pH 7.0, and D-lactate oxidation was not detectable. The Gln-296 enzyme showed constant pyruvate K_M values of about 100 mM, and highly pH-dependent k_{cat} values, which were virtually proportional to the proton concentration in the solvent (Fig. 1).

Effect of Glu-264 Substitution on the pH-Profile of the Catalysis—Kochhar *et al.* reported that the replacement of Glu-264 with Gly in *L. bulgaricus* D-LDH decreased the pK of the catalytic His by two pH units, but did not markedly reduced the catalytic activity, concluding that Glu-264, unlike Asp-168 of L-LDH, only modulates the pH dependence of the catalytic activity through its negative charge that is closely linked to the catalytic His imidazole (*21*). Nevertheless, the substitution of Gly may induce a large structural change in the active site through complete loss of the side chain. In this study, therefore, Glu-264 was

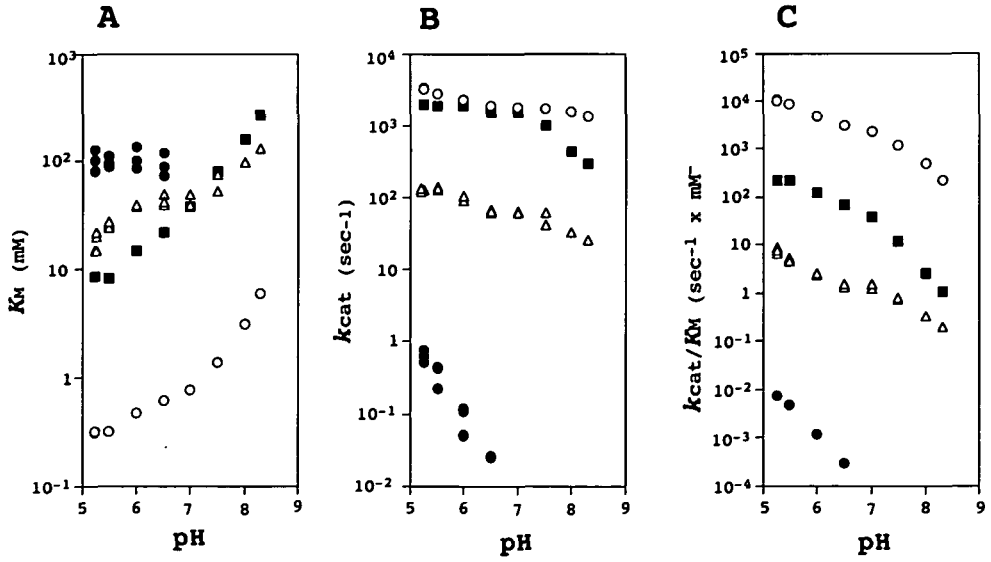

Fig. 1. **pH dependence of kinetic parameters for pyruvate reduction by the wild-type (○), Gln-296 (●), Asp-264 (■), and Gln-264 (△) enzymes.** The buffers used for the assay were 50 mM sodium Mes (pH 5.2 to 6.5), sodium Mops (pH 7.0), and sodium Hepes (pH 7.5 to 8.3). Panel A, K_M *versus* pH; panel B, k_{cat} *versus* pH; panel C, k_{cat}/K_M *versus* pH.

replaced with Gln in the *L. pentosus* enzyme to evaluate the crucial role of Glu-264. Since Gln has, besides a similar sized side chain to Glu, the potential to form a hydrogen bond like Glu, the substitution of Gln may minimize the structural change of the protein. Actually, the corresponding Glu residue is replaced by Gln in the case of FDH (*7*). To evaluate the exact role of the carboxyl group on its side chain, Glu-264 was also replaced with Asp, another acidic amino acid.

In pyruvate reduction, the wild-type enzyme shows a virtually constant k_{cat}, but a highly pH-dependent pyruvate K_M above pH 7.0, the pK of the His-296 imidazole (*13, 14*). In contrast, the Gln-264 enzyme displayed less significant pH dependence of the pyruvate K_M (Fig. 1, panel A), but slightly increased pH-dependence of the k_{cat} (Fig. 1, panel B). In the pH range of 5.2 to 8.5, the Gln-264 enzyme exhibited a more than 10-fold higher pyruvate K_M and a more than 10-fold lower k_{cat} than the wild-type enzyme, resulting in a k_{cat}/K_M of about 10^3-fold lower (Fig. 1). Below pH 7.0, on the other hand, the Asp-264 enzyme displayed similar shaped pH profile curves for K_M, k_{cat}, and k_{cat}/K_M to those of the wild-type, though the k_{cat} were obviously reduced above pH 7.5. Below pH 7.0, pyruvate K_M was more than 10-fold higher than that of the wild-type enzyme, but k_{cat} was reduced by only a factor of 2, and k_{cat}/K_M by only 10 to 10^2.

In D-lactate oxidation, the wild-type enzyme exhibits greatly increased D-lactate K_M values below pH 7.0, since the deprotonated imidazole of His-296 is required for the D-lactate binding (*13*). In the pH range of 6.0 to 8.5, in contrast, the Gln-264 enzyme showed an apparently constant K_M of about 80 mM and highly pH-dependent k_{cat} throughout this pH range, exhibiting a k_{cat}/K_M reduced by more than 10^3 (Fig. 2). The Asp-264 enzyme also exhibited a constant D-lactate K_M of about 100 mM, and a highly pH-dependent k_{cat} below pH 7.0. Nevertheless, this enzyme showed a virtually constant k_{cat} above pH 7.5 and a k_{cat}/K_M reduced by only about 10^2.

Effect of Substitution of Glu-264 on the pK of His-296 Imidazole—Unlike the case of the *L. bulgaricus* Gly-264 mutant D-LDH (*21*), the pH profiles for K_M and k_{cat}/K_M did not clearly show the pK of the His-296 imidazole in the Gln- and Asp-264 enzymes (Figs. 1 and 2). To determine the exact pK values, therefore, the enzymes were treated with diethylpyrocarbonate (DEPC) under various pH conditions in the absence and presence of NADH (1 mM) (Fig. 3). Since the inactivation rate is in proportion to the concentration of DEPC and unprotonated catalytic His, the pseudo-first-order rate constant (k_{app}) and the pK of the catalytic His depend on the following equation.

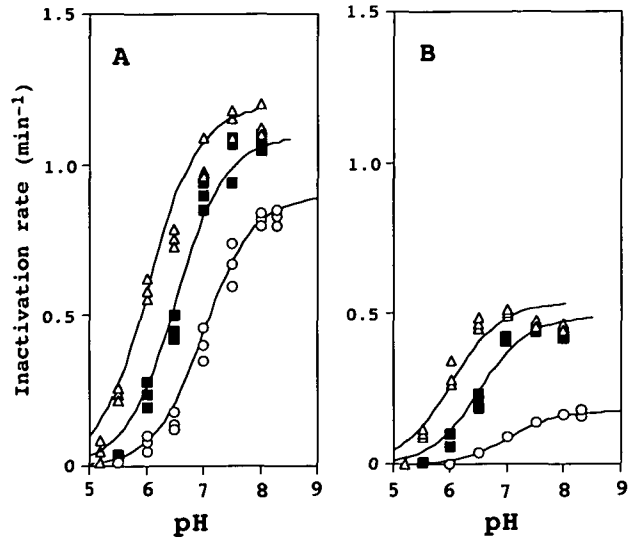

Fig. 3. **pH dependence of DEPC-induced inactivation of the wild-type (○), Asp-264 (■), and Gln-264 (△) enzymes in the absence (panel A) and presence (panel B) of a saturation level of NADH.** The enzymes (10 μM) were treated with 0.12 mM DEPC at 30°C in 100 mM sodium Mes (pH 5.2 to 6.5), sodium Mops (pH 7.0), and sodium Hepes (pH 7.5 to 8.3) buffers without (A) or with (B) 1 mM NADH. Aliquots of the wild-type enzyme were diluted with the Hepes buffer, pH 7.0, containing 2.5 mM histidine at intervals, then the remaining activity was measured in 50 mM sodium Mes buffer, pH 5.5. Aliquots of the mutant enzymes were directly assayed in the same buffer as in the case of the wild type enzyme at intervals. The reciprocals of the half lives of the enzyme are plotted *versus* pH. Solid lines indicate the calculated curves for inactivation rate (see text).

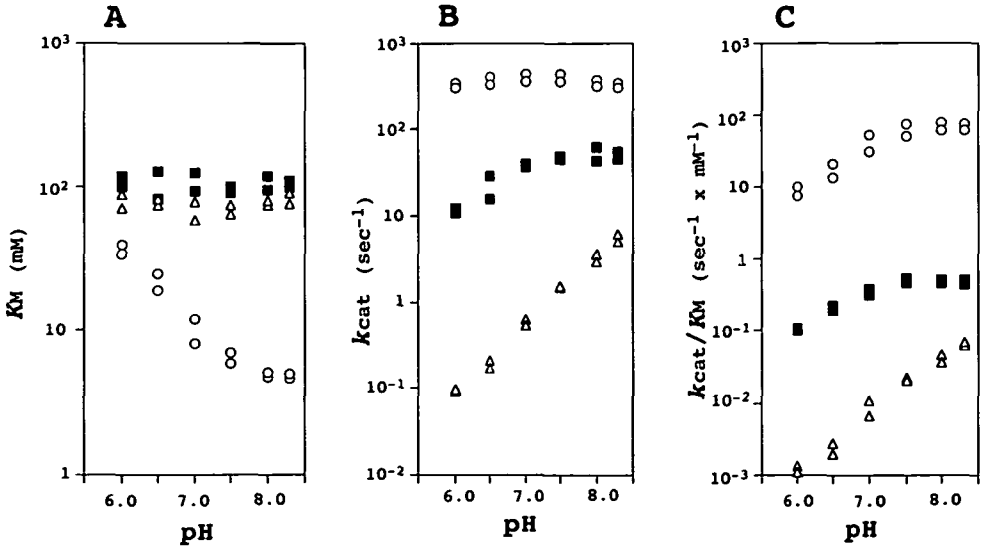

Fig. 2. **pH dependence of kinetic parameters for in D-lactate oxidation by the wild-type (○), Asp-264 (■), and Gln-264 (△) enzymes.** The buffers used for the assay were 50 mM sodium Mes (pH 5.2 to 6.5), sodium Mops (pH 7.0), and sodium Hepes (pH 7.5 to 8.3). Panel A, K_M *versus* pH; panel B, k_{cat} *versus* pH; panel C, k_{cat}/K_M *versus* pH.

$k_{app} = k_{appmax}/\{1 + [H^+]/K_a\}$, where $pK = -\log K_a$

The His pK values are calculated as 7.0, 6.0, and 6.5, and k_{appmax} values are 0.9, 1.2, and 1.1, in the absence of NADH, and 0.18, 0.55, and 0.55 in the presence of NADH, for the wild-type, Gln-264 and Asp-264 enzymes, respectively, from the intercept and the slope of the plot $1/k_{app}$ *versus* $[H^+]$. Figure 3 also indicates the calculated curves for the inactivation rate. Thus, the Gln- and Asp-264 enzymes displayed a greatly shifted pH dependence of the drug-sensitivity to the acid side, together with markedly increased k_{appmax}.

Protective Effects of NADH and Oxamate on DEPC-Induced Inactivation of the Mutant Enzymes—Besides shifts in the pH-dependence, the Glu-264 substitutions also induced significant increases in the DEPC-sensitivity of 1.4- and 3-fold in the absence and presence of 1 mM NADH, respectively (Fig. 3), unlike Arg-235 substitutions, which induced a shift of the His pK, but no significant change in the drug-sensitivity (*14*). Together with the wild-type, and Lys- and Gln-235 enzymes (*14*), the Gln- and Asp-264 enzymes were treated with DEPC in the presence of various concentrations of NADH (Fig. 4, panel A). Essentially an equivalent dose of NADH sufficiently gave the saturation level for the protection of all the enzymes (Fig. 4, panel A), indicating that neither Glu-264 nor Arg-235 substitutions induced significant reduction in the NADH-binding. However, the Glu-264 mutant enzymes were much less protected by the saturation level of NADH than the other enzymes (Figs. 3 and 4).

In the presence of the saturation level of NADH (1 mM), on the other hand, oxamate protected the Arg-235 and Glu-264 mutant enzymes from the DEPC-induced inactivation at pH 7.5 and 6.5 less than the wild-type enzyme (Fig. 4, panels B and C). The protective effect of oxamate on the wild-type enzyme was greatly affected by the pH more than the effect on the mutant enzymes. Consequently, oxamate protected the mutant enzymes much less, even at pH 6.5, than the wild-type enzyme at pH 7.5 (Fig. 4, panels B and C), indicating that the apparent loss of oxamate binding, if any, is not only due to the decreased His pK values of the mutant enzymes.

Inhibitory Effects of Carboxylates on the Wild-Type and Mutant D-LDHs—A 2-ketoacid such as oxamate, and non-2-ketoacids such as formate, acetate, and propionate inhibited the pyruvate reduction by the wild-type and mutant enzymes, competing with the substrate pyruvate (data not shown). To evaluate the imidazole-carbonyl interaction, we compared the inhibitory effects of these carboxylates on the wild-type and mutant enzymes at pH 5.5 (Table I), where His-296 is, if present, mostly protonated (Fig. 3) (*14*). In the case of the wild-type enzyme, oxamate showed a more than 10-fold smaller inhibition constant (K_I) than the other acids. In the case of the Gln-296 enzyme, in contrast, oxamate gave a 30-fold higher K_I, but formate and acetate gave only 2- or 3-fold higher K_I values, indicating that the inhibitor binding is also significantly stabilized through the interaction between the His imidazole and the ligand carbonyl group.

The replacement of Arg-235 with Gln reduced the inhibitory effects of oxamate, formate, acetate, and propionate by 140, 15, 7, and 3 times, respectively, with concomitant

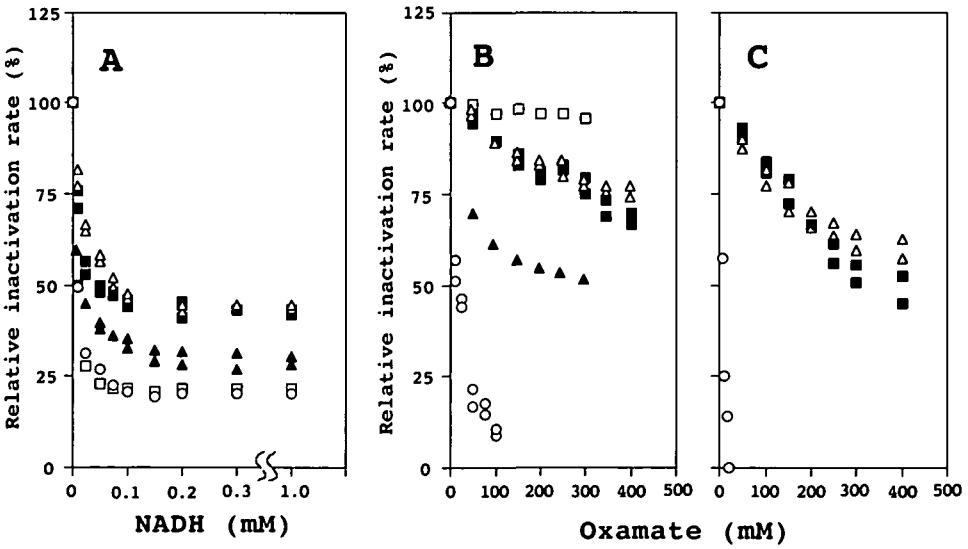

Fig. 4. **Protective effects of NADH (panel A) and oxamate (panels B and C) on inactivation of the wild-type (○), Asp-264 (■), Gln-264 (△), Lys-235 (▲), and Gln-235 (□) enzymes by DEPC.** The enzymes were treated with 0.12 mM DEPC at 30°C in 100 mM sodium Hepes (pH 7.5) (A and B) and sodium Mes (pH 6.5) (C) buffers in the presence of the indicated concentrations of NADH (A), or 1 mM NADH plus the indicated concentrations of oxamate (B and C), then the inactivation rates were determined as in Fig. 3. The inactivation rates are plotted as relative rates to those in the absence of NADH (A), or oxamate (B and C).

TABLE I. **Inhibition constants (mM) for competitive inhibitors.**

Enzyme	Oxamate NH_2COCOO^-	Formate $CHOO^-$	Acetate CH_3COO^-	Propionate $CH_3CH_2COO^-$
Wild-type	4.0	70	65	50
H296Q	120	170	170	50
R235Q	550	1,050	450	150
R235K	55	80	75	35
E264Q	40	60	45	30
E264D	25	55	65	30

[a]The enzyme assay was performed at pH 5.5 in 100 mM sodium Mes buffer. [b]Mutant enzymes were named using the single-letter amino acid code, indicating the number of the residue (*e.g.* H296Y, His-296 to tyrosine, *etc.*).

loss of the predominant effect of oxamate (Table I). On the other hand, the Arg-235 to Lys substitution induced a further specific reduction in the oxamate inhibition by 14-fold, but caused no marked reduction in the others. The Glu-264 to Gln and Asp substitutions also specifically decreased the inhibitory binding of oxamate by 10- and 6-fold, respectively, and induced no change or even an increase in the inhibition by the other acids.

The K_I values for 2-keto and non-2-ketoacids were compared at various pHs with the wild-type and mutant enzymes (Fig. 5). In the case of the wild-type enzyme (Fig. 5, panel A), propionate, a non-2-ketoacid, also showed a significant pH-dependence of the K_I value, indicating that pH significantly affect the ligand binding even if the imidazole-carbonyl interaction is absent. Nevertheless, oxamate displayed significantly higher pH-dependence than propionate above pH 7.0, which is in good agreement with the pK value of His-296 (Fig. 3).

In the case of the Lys-235 enzyme (Fig. 5, panel B), which has the same His-296 pK as the wild-type enzyme (*14*), oxamate exhibited a rather low inhibitory effect on the enzyme reaction compared to propionate throughout the pH range of 5.2 to 8.3. In this case, oxamate and propionate showed no marked difference in the pH-dependence, and displayed the same shaped pH profile curve as did propionate for the wild-type enzyme (Fig. 5, panels A and B). The Gln-264 enzyme also showed a reduced pH-dependence of the oxamate K_I, and in this case, oxamate even displayed a rather small pH-dependence compared to propionate (Fig. 5, panel C). In the case of the Asp-264 enzyme, on the other hand, oxamate exhibited a slightly higher inhibitory effect than propionate below pH 7 but a lower effect above pH 7.0, and thus consequently displayed a slightly but significantly higher pH dependence than propionate (panel D). In this case, the oxamate and propionate K_I ratio apparently showed two constant values under acidic and alkaline conditions, giving two plateaus on the curve, and was shifted between the two values in the pH range of 6.0 to 7.5 (Fig. 3).

Catalytic Constants of the Wild-Type and Mutant D-*LDHs*—Table II summarizes the catalytic constants for the wild-type and mutant enzymes with respect to pyruvate reduction at pH 5.5, where His-296, if present, is mostly protonated. The Glu-264 to Gln substitution, though much less so than the His-296 and Arg-235 substitutions, greatly reduced the enzyme activity, inducing an about 90-fold increase in the pyruvate K_M and a 20-fold reduction in the k_{cat}. On the other hand, the Gln to Asp substitution induced only a 30-fold increase in the pyruvate K_M and a 1.5-fold reduction in the k_{cat}.

Using a deuterium derivative of NADH (NADD), catalytic constants were determined, and compared with those for NADH through steady-state kinetics. In all the enzyme reactions, the deuterium on the coenzyme did not significantly affect the K_M values for coenzyme and substrate (data not shown), but markedly reduced the k_{cat} values. Table II also shows the primary isotope effects (k_{cat} for NADH *versus* k_{cat} for NADD) on the wild-type and mutant enzymes. For the wild-type enzyme, the reaction rate was slightly reduced (1.6-fold) when NADD was used as a coenzyme, suggesting that the hydrogen transfer is one of

TABLE II. **Kinetic constants for pyruvate reduction by** D-**LDHs.**

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1} \cdot s^{-1}$)	v_{NADH}/v_{NADD}
Wild-type	0.3	2,800	8,700	1.6
H296Q	100	0.44	0.0044	2.0
R235Q	180	10	0.06	2.5
R235K	35	16	0.46	2.6
E264Q	26	130	5	2.3
E264D	8.5	1,900	220	2.3

The assay conditions and names of mutant enzymes are given in Table I.

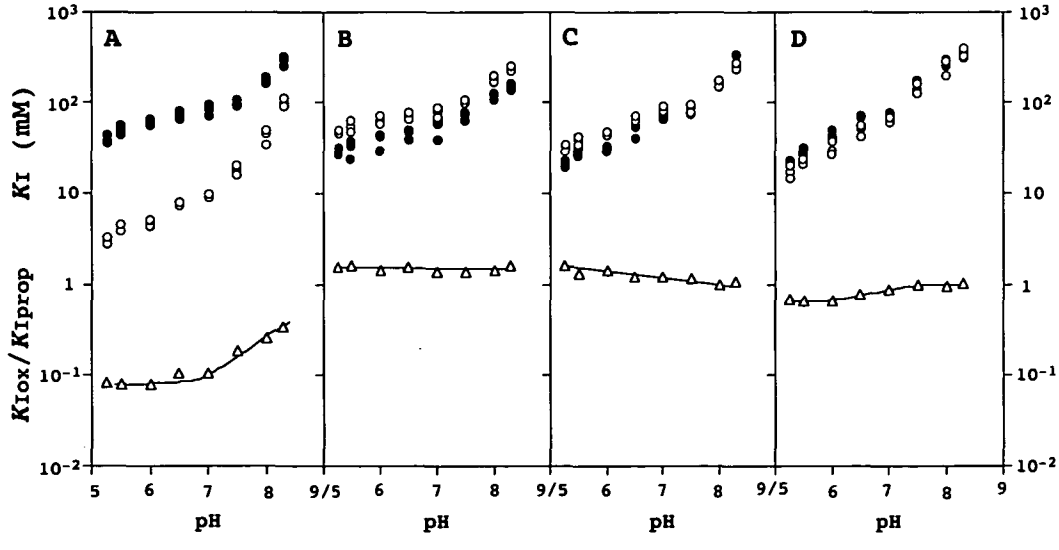

Fig. 5. **pH dependence of K_I for oxamate (○) and propionate (●), and the ratio of oxamate K_I *versus* propionate K_I (△) for the wild-type (panel A), Lys-235 (panel B), Gln-264 (panel C), and Asp-264 (panel D) enzymes.** The buffers used for the assay were 50 mM sodium Mes (pH 5.2 to 6.5), sodium Mops (pH 7.0), and sodium Hepes (pH 7.5 to 8.3). The inhibition constants were determined through three independent experiments at each pH, and the ratio of the inhibition constants (oxamate K_I/propionate K_I) was calculated from the mean K_I values.

the slow steps in the catalysis, though it is possibly not a fully rate-limiting step. On the other hand, however, the Gln-296 enzyme showed a significantly increased isotope effect up to 2.0, and the Lys- and Gln-235, and Gln- and Asp-264 enzymes exhibited further markedly increased isotope effects up to 2.6 and 2.3, respectively.

DISCUSSION

Although no ternary complex structure of D-2-hydroxyacid dehydrogenases, except for the case of FDH, has yet been determined, Stoll *et al.* proposed a possible model of the D-LDH active site using *L. pentosus* apo-D-LDH structure as a guide (*19*). According to this model, Arg-235 not only anchors the substrate carboxyl group like Arg-171 of L-LDH, in support of the Val-78-Gly-79 backbone atoms, but also fulfils the role of Arg-109 of L-LDH, through bidentate hydrogen bonds with the carboxyl and carbonyl groups of pyruvate. The model of "bifunctional" Arg-235 was supported by the following two lines of evidence. 1. There is no other possible candidate for Arg-109 as yet in the D-2-hydroxyacid dehydrogenase family. 2. The Arg-235 substitutions not only induced an increase in the substrate K_M but also a great reduction in the k_{cat} (*14*). Furthermore, this model is strongly supported by the results of the present study.

While the Lys to Gln substitution markedly reduced the binding of all the acid inhibitors tested, the Arg-235 to Lys substitution only diminished the oxamate binding, but not the others (Table I), indicating that Lys can also stabilize the binding of the ligand carboxyl group instead of Arg. Although it is uncertain how the active site structure is changed through this substitution, the structure may be flexible enough to orient the ε-amino group of Lys toward the ligand carboxyl group. Nevertheless, Lys appears to fulfil only one of the "bifunctional" roles of Arg, *i.e.*, anchoring the substrate carboxyl group. The Arg to Lys substitution, together with the Arg to Gln one, more greatly destabilized the inhibitory binding of oxamate than those of non-2-keto acids (Table I), and also induced a great loss of pH dependence in the oxamate inhibition. In addition, the substitutions markedly increased the primary isotope effect on the enzyme catalysis (Table II). These results indicate that Arg-235 not only anchors the ligand carboxyl group, but also supports the interaction between the catalytic His imidazole and the ligand carbonyl oxygen, enhancing the substrate binding and the hydrogen transfer step in the catalysis. Through the bidentate interaction, therefore, the guanidino group of Arg-235 appears to allow the substrate to be correctly orientated and sufficiently polarized in the active site, like those of Args-171 (*15*) and 109 (*19*) in L-LDH, respectively, so that the substrate carbonyl oxygen can efficiently accept the imidazole proton of His-296 (Fig. 6).

The replacement of Glu-264 with Gln induced an about 90-fold increase in K_M and a 20-fold reduction in k_{cat} even under conditions where the His-296 was mostly protonated (Table II), indicating that Glu-264 does not simply modulate the pH-dependence of the enzyme activity. When the change in the free energy of transition state binding (ΔG) is calculated as $\Delta G = RT \ln [(k_{cat}/K_M \text{ of the mutant enzyme})/(k_{cat}/K_M \text{ of the wild-type enzyme})]$, the change in the k_{cat}/K_M value with the Glu to Gln substitution (1,740-fold reduction) corresponds to a ΔG change of -4.5 kcal/mol. In the case of L-LDH, the Asp-168 to Asn substitution induced a 2,100-fold reduction of the k_{cat}/K_M value (*18*), corresponding to a ΔG change of -4.6 kcal/mol. The good agreement of ΔG indicates that Asp-168 and Glu-264 are of equivalent significance in stabilization of the transition state complex. In addition, the Glu-264 substitution also induced the loss of the imidazole-carbonyl interaction in the 2-keto acid binding (Table I and Fig. 5), and increase in the primary isotope effect on the catalysis (Table II). We concluded, therefore, that Glu-264 also supports the His-296 function in the substrate binding and catalysis, as in the case of Arg-235. On the other hand, the substitution of Asp, another acidic amino acid, induced only a 40-fold reduction in k_{cat}/K_M (Table II), *i.e.*, a ΔG change of -2.2 kcal/mol, and less reduced affinity to 2-keto acid ligands than the Glu to Gln substitution (Fig. 5), indicating that the negative charge is actually a pivot in the Glu-264 function.

In the case of FDH, the conserved Glu in the D-2-hy-

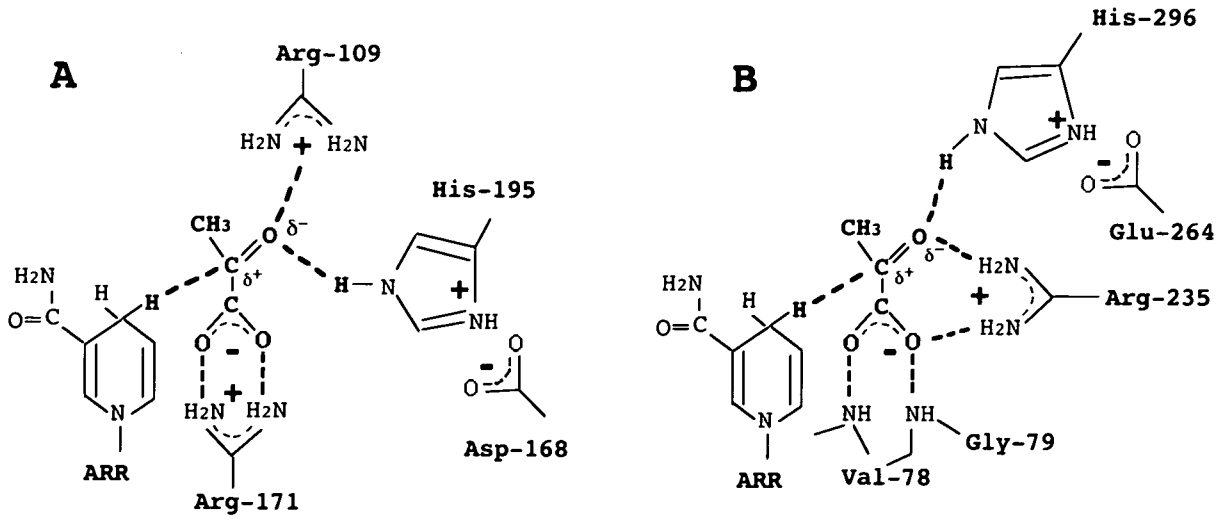

Fig. 6. **Comparison of the possible active site of D-lactate dehydrogenase ternary complex (B) with the L-lactate dehydrogenase active site (A) in the ternary complexes.**

droxyacid dehydrogenase family is uniquely replaced with Gln (*7*), and no great change in the FDH activity is induced by the replacement of the His-Gln pair with His-Glu through site-directed mutagenesis, except for a marked reduction in the pH range for the substrate binding (*27*). These findings are, however, not surprising. Since FDH mediates no proton transfer during its catalytic reaction, unlike the other enzymes, the role of the conserved His must be different in FDH from that in the D-2-hydroxyacid dehydrogenases.

It is, thus, strongly suggested that Arg-235 and Glu-264 fulfil the roles of Arg-109 and Asp-168 in L-LDH, respectively, and cooperatively stabilize both the enzyme-substrate and transition state complexes. Nevertheless, the amino acid substitutions also gave some obviously different results in the two LDHs. In the case of L-LDH, the Asp-168 to Asn substitution more severely decreased the pyruvate binding than the L-lactate binding, suggesting that the negative charge is more highly required in the pyruvate binding to neutralize the protonated His imidazole, than in the L-lactate binding, where the imidazole is deprotonated (*18*). In D-LDH, however, the Glu-264 to Gln substitution apparently induced the same level of damage to the forward and reverse reactions (Figs. 1 and 2). The protonated imidazole may have to be neutralized only under the highly positive environment in the L-LDH active site, where Arg-109 and Arg-171 are involved besides the imidazole, but not in the less charged D-LDH active site, where only one Arg, Arg-235, is involved (Fig. 6).

In the case of L-LDH, the Asp-168 to Asn substitution, as well as the Arg-109 to Gln one (*19*), did not markedly affect the His pK of the L-LDH holo-enzyme. In L-LDH, the substrate binding induces great rearrangement of the L-LDH active site (*28*). The rearrangement apparently not only allows Arg-109 to come near His-195, but also Asp-168 to form a strong ionic interaction with His-195 through the desolvation of the active site (*18*). In contrast, however, the substitution of Glu-264 with Gln, even with Asp, greatly influenced the His-296 pK values of the apo- and holo-D-LDHs (Fig. 3), as in the case of the Arg-235 to Gln substitution (*14*). Besides the shift of the His pK, the Glu-264 substitutions also significantly increased reactivity of the deprotonated His-296 to DEPC in both the apo-enzyme and, in particular, the holo-enzyme (Fig. 3), suggesting that Glu-264 greatly affects the His imidazole. These results indicate that the negative charge of Glu-264, together with the positive one of Arg-235, is closely linked to His-296 even in the apo- and holo-enzymes, as shown in D-LDH (*9*) and D-glycerate dehydrogenase (*11*) apo-enzyme structures, and may alow the His imidazole to bind the substrate in the active site, although the substrate binding may induce the marked conformational change also in D-type of dehydrogenases (*10*). To further understand the catalytic mechanism of D-LDH, it is desirable to analyze the ternary complex enzyme structure and the transition state in the enzyme catalysis.

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