J. Biochem. **138**, 741–749 (2005)
doi:10.1093/jb/mvi170

Recognition Site for the Side Chain of 2-Ketoacid Substrate in D-Lactate Dehydrogenase

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Received July 28, 2005; accepted September 8, 2005

Replacement of Tyr52 with Val or Ala in *Lactobacillus pentosus* D-lactate dehydrogenase induced high activity and preference for large aliphatic 2-ketoacids and phenylpyruvate. On the other hand, replacements with Arg, Thr or Asp severely reduced the enzyme activity, and the Tyr52Arg enzyme, the only one that exhibited significant enzyme activity, showed a similar substrate preference to the Tyr52Val and Tyr52Ala enzymes. Replacement of Phe299 with Gly or Ser greatly reduced the enzyme activity with less marked change in the substrate preference. Except for the Phe299Ser enzyme, these mutant enzymes with low catalytic activity consistently stimulated NADH oxidation in the absence of 2-ketoacid substrates. However, the double mutant enzymes, Tyr52Arg/Phe299Gly and Tyr52Thr/Phe299Ser, did not exhibit synergically decreased enzyme activity or the substrate-independent NADH oxidation, but rather increased activities toward certain 2-ketoacid substrates. These results indicate that the coordinative combination of amino acid residues at two positions is pivotal in both the functional recognition of the 2-ketoacid side chain and the protection of the bound NADH molecule from the solvent. Multiplicity in such combinations appears to provide D-LDH–related 2-hydroxyacid dehydrogenases with a great variety of catalytic and physiological functions.

Key words: enzyme catalysis, 2-hydroxyacid dehydrogenase, D-lactate dehydrogenase, substrate specificity.

Abbreviations: D-GDH, D-glycerate dehydrogenase; D-HicDH, D-2-hydroxyisocaproate dehydrogenase; 2-HydDH, 2-hydroxyacid dehydrogenase, D-LDH, D-lactate dehydrogenase; D-PGDH, D-3-phosphoglycerate dehydrogenase; MES, 2-(*N*-morpholino)ethane sulfonic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

NAD-dependent D-lactate dehydrogenase (D-LDH, EC 1.1.1.28), which converts pyruvate into D-lactate and oxidizes NADH into NAD^+ (*1*), is evolutionally separate from NAD-dependent L-lactate dehydrogenase (L-LDH, EC 1.1.1.27) in spite of the similarity in their catalytic reactions (*2–4*). D-LDH shares a common protein structure with many other 2-hydroxyacid dehydrogenases (2-HydDH), which commonly catalyze the oxidation-reduction between 2-ketoacids and 2-hydroxyacids (all D-isoforms except for glyoxylate reductase), together with some other dehydrogenases such as formate (*5–7*), L-alanine (*8*) and phosphite dehydrogenases (*9*, *10*). The D-LDH–related 2-HydDHs comprise various enzymes that exhibit different substrate specificities and physiological functions, such as D-3-phosphoglycerate (*11*, *12*), D-glycerate (*13*, *14*), D-2-hydroxyisocaproate (*15*) dehydrogenases (D-PGDH, D-GDH and D-HicDH, respectively), glyoxylate reductase (*16*, *17*), and vancomycin-resistant protein H (VanH) (*18*), which prefers pyruvate or larger aliphatic 2-ketoacids as substrates (*19*), and transcryptional co-repressor CTBP (*20*, *21*), which weakly catalyzes the conversion of pyruvate to lactate (*21*). Structural and biochemical studies on D-LDHs strongly suggest that these 2-HydDHs are equipped with essentially the same catalytic machinery except for the recognition for the distinct side chains of 2-ketoacid or 2-hydroxyacid substrates (*22–27*).

Substrate recognition in L-LDH has been extensively studied by means of protein engineering (*28–34*), but much less is known about D-LDH. The three-dimensional structures of D-LDHs (*25*, *35*) and D-HicDH (*26*) imply that the side chains of Tyr52 and Phe299 of *Lactobacillus pentosus* D-LDH can be located very near the side chain of the 2-ketoacid substrate (Fig. 1), and are thereby directly involved in the recognition of the substrate side chains. Actually, the single amino acid replacement of Tyr52 with Leu sufficiently converts *L. pentosus* D-LDH into a D-HicDH (*36*). While the known D-2-HydDHs that prefer aliphatic 2-ketoacids possess aromatic or aliphatic amino acids at position 52 and aromatic amino acids at position 299, D-GDH and D-PGDH have polar or positively charged amino acids at position 52, and small amino acids such as Gly and Ser at position 299 (Fig. 2). In this study, we characterized mutant *L. pentosus* D-LDHs in which Tyr52 or Phe299 was replaced with various amino acids, in order to evaluate crucial roles of these amino acid residues in the function of the *L. pentosus* enzyme and related 2-HydDHs.

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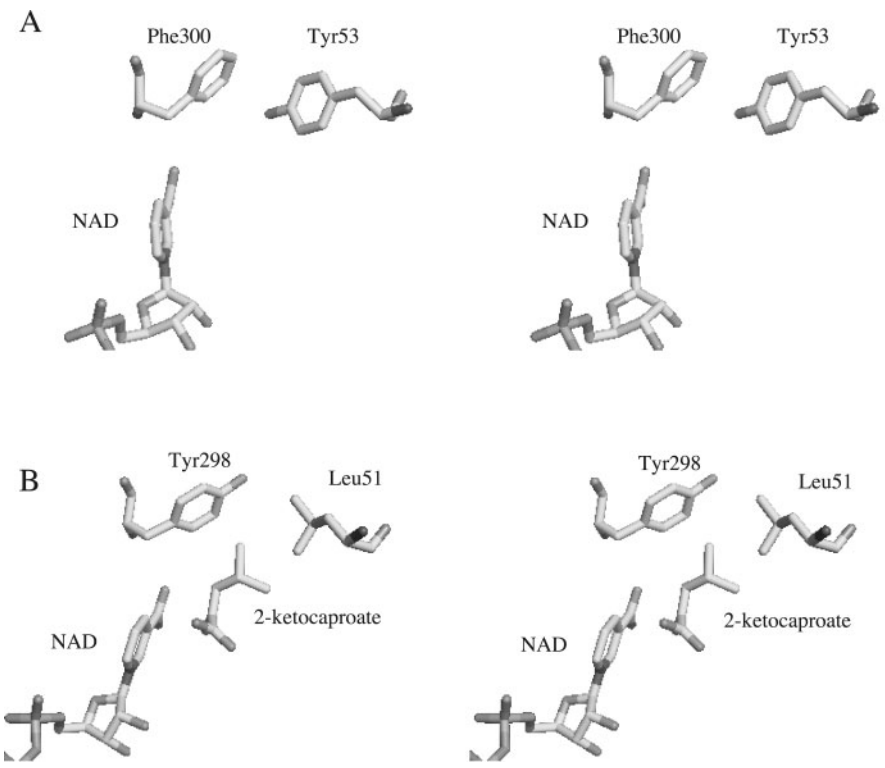

Fig. 1. **Stereo diagrams of the substrate binding site in *L. bulgaricus* D-LDH (panel A) and *L. casei* D-HicDH (panel B) for the 2-ketoacid side substrate chain.** The figure was drawn by using PyMol (*44*), according to the substrate binding sites of the *L. bulgaricus* D-LDH binary complex (2DLD) (*35*) and *L. casei* D-HicDH ternary complex (1DXY) (*26*) structures. The diagrams show only the bound ligands, NAD and 2-ketoisocaproate, and only the relevant amino acid residues, Tyr53 and Phe300 of the *L. bulgaricus* enzyme, and Leu51 and Tyr298 of *L. casei* enzyme, which correspond to Tyr52 and Phe299 of *L. pentosus* D-LDHs, respectively.

A

LPLDH	47	DGADV**Y**QQKDYT	58
LBLDH	48	DGVVV**Y**QQLDYT	59
LDLDH	47	DGVVV**Y**QQLDYT	58
LJLDH	48	DGVVV**Y**QQLDYT	59
PALDH	46	DGVVA**Y**QQKPYT	57
LMLDH	47	DSAVV**Y**QQLDYT	58
SALDH	47	DGVTT**M**QFGKLE	58
ECLDH	46	EAVCI**F**VNDDGS	57
LCHDH	46	DGINS**L**QTTPYA	57
LDHDH	47	SSV-S**L**KPLGPV	57
EFVRH	49	QCI-S**V**GMKSEI	59
ECPGDH	54	HFIGL**R**SRTHLT	65
MTPGDH	45	DALLV**R**SATTVD	56
HMGDH	47	DALLI**T**LNEKCR	58

B

LPLDH	293	ITPHTA**F**YTETAVH	306
LBLDH	294	VTPHTA**F**YTTHAVR	307
LDLDH	293	VTPHTA**F**YTTHAVR	306
LJLDH	294	VTPHTA**F**YTTHAVR	307
PALDH	292	ITPHAA**F**YTRPAVK	305
LMLDH	293	ITPHTA**F**YTTKAVL	306
SALDH	293	VTPHIA**F**FSDEAVQ	306
ECLDH	292	FTGHQA**F**LTAEALT	305
LCHDH	292	LSPHIA**Y**YTETAVH	305
LDHDH	295	ITPHSA**F**YTETSIR	308
EFVRH	289	ITPHTA**Y**YTEQALR	302
ECPGDH	289	LTPHIG**G**STQEAQE	302
MTPGDH	277	VTPHLG**A**STAEAQD	290
HMGDH	283	LFPHIG**S**AATQARE	296
CSGDH	317	IVPHIA**S**ASKWTRE	330
TLGR	285	LAPHIG**S**ATHEARE	298
HSHPGR	287	ILFHIG**S**ATHRTRN	300
HSCTBP	311	CTPHAA**W**YSEQASI	324

Fig. 2. **Amino acid sequences in the regions around the amino acids corresponding to Tyr52 (panel A) and Phe299 (panel B) of *L. pentosus* D-LDH.** LPLDH, *L. pentosus* D-LDH (*2*); LBLDH, *L. bulgaricus* D-LDH (*4*); LDLDH, *L. delbruekii* D-LDH (*3*); LJLDH, *L. johnsonii* D-LDH (*45*); PALDH, *Pediococcus acidilactici* D-LDH (*46*); LMLDH, *Leuconostoc mesenteroides* D-LDH (*47*); SALDH, *Staphylococcus aureus* D-LDH (*48*); ECLDH, *Escherichia coli* D-LDH (*49*); LCHDH, *L. casei* D-HicDH (*15*); LDHDH, *L. delbruekii* D-HicDH (*50*); EFVRH, *Enterococcus faecium* vancomycin resistant protein VanH (*18*); ECPGDH, *E. coli* D-3-phosphoglycerate dehydrogenase (*11*); MTPGDH, *Mycobacterium tuberculosis* D-3-phosphoglycerate dehydrogenase (*43*); HMGDH, *Hyphomicrobium methylovorum* D-glycerate dehydrogenase (*14*); CSGDH, *Cucumis salivus* (cucumber) D-glycerate dehydrogenase (*13*); TLGR, *Thermococcus litoralis* glyoxylate reductase (*16*); HSHPGR, *Homo sapience* hydroxypyruvate/glyoxylate reductase (*17*); HSCTBP, *Homo sapiens* transcriptional co-repressor CTBP (*20*); The asterisks indicate the positions corresponding to Tyr52 and Phe299 of *L. pentosus* D-LDH. The sequences around position 52 are not indicated in the cases of CSGDR, TLGR, HSHPGR and HSCTBP, since they are too different from the sequences of the other proteins to be correctly aligned.

MATERIALS AND METHODS

Amino Acid Replacement—Table 1 lists the synthetic oligodeoxynucleotides used for site-directed mutagenesis in this study. The replacement of Tyr52 with Val, Ala, Thr, Asp or Arg was performed with a Gene Editor (Promega) to produce the Y52V, Y52A, Y52T, Y52D and Y52R mutant enzymes, respectively. The replacement of Phe299 with Gly and Ser was done with a MUTA-GENE in vitro mutagenesis kit (Bio-Rad) for the F299G and F299S

Table 1. **Nucleotide sequences of synthetic oligonucleotides used for site-directed mutagenesis.**

F299G	5′-CATACGGCTGGCTACACTGA-3′
F299S	5′-CATACGGCTAGCTACACTGA-3′
Y52T	5′-CGGTGCCGATGTAACGCAACAAAAG-GACTATACTGC-3′
Y52R	5′-CGGTGCCGATGTAAGGCAACAAAAG-GACTATACTGC-3′
Y52V	5′-CGGTGCCGATGTAGTCCAACAAAAG-GACTATACTGC-3′
Y52A	5′-CGGTGCCGATGTAGCCCAACAAAAG-GACTATACTGC-3′
Y52D	5′-CGGTGCCGATGTAGACCAACAAAAG-GACTATACTGC-3′

enzymes, according to Kunkel (*37*). The DNA fragments were sequenced by the dideoxy chain terminator procedure (*38*) with a DNA sequencer model 4000L (LI-COR) to prove that only the desired mutation had occurred.

Enzyme Preparation—The *L. pentosus* recombinant D-LDHs were produced in *Escherichia coli* MV1184 cells harboring expression plasmids for the corresponding enzyme genes and purified essentially as described previously (*22*, *23*). The purity of the enzyme preparations was examined by SDS-PAGE according to Laemmli (*39*).

Enzyme Assay and Protein Determination—The enzyme assay was performed at 30°C in 100 mM sodium MES [2-(*N*-morpholino)ethane sulfonic acid] buffer (pH 5.5) containing 0.01 to 10 μM of the enzymes, 0.1 mM NADH, which was the saturation level for all the enzymes used in this study, and various concentrations of 2-ketoacids (sodium salts). Reaction velocity (v) was defined as the rate of NADH oxidation (s^{-1}) by one subunit of the enzyme. Kinetic parameters were calculated from plots of v/[S] *versus* [S]. The change in the free energy of transition-state binding (ΔG) was calculated from $\Delta G = RT\ln[(k_{cat}/K_m$ of the mutant enzyme)/(k_{cat}/K_m of the wild-type enzyme)]. The deuterium derivative of NADH [NADD, (nicotinamide-4-^{2}H)NADH] was prepared according to Colowick and Kaplan (*40*) to be used for the determination of primary isotope effects on the 2-ketoacid reduction by the wild-type and mutant D-LDHs. Protein concentrations were calculated using extinction coefficients at 280 nm of 27,045 $M^{-1}\cdot cm^{-1}$ for D-LDHs, as determined from the amino acid compositions and molecular weights of *L. pentosus* D-LDH (*2*).

NADH Binding to D-LDHs—The dissociation constants (K_d) of D-LDH and NADH were determined essentially according to the previous report (*36*). The binding of NADH to the D-LDHs was followed as the change in NADH fluorescence intensity (ΔF) essentially according to the method used for L-LDHs (*41*), with excitation and emission wavelengths of 340 and 460 nm, respectively, using a JASCO FP-750 spectrofluorophotometer. ΔF was determined by comparing the fluorescence intensities of NADH in the presence and absence of the enzymes (15 μM) at 30°C in 50 mM sodium MES buffer (pH 6.0). The K_ds for the enzymes with NADH were calculated according to the procedure for L-LDHs (*36*, *41*) by curve-fitting with KaleidaGraph.

Determination of Substrate-Independent NADH Oxidation Rate—Time-dependent change in fluorescence intensity of NADH without substrate was monitored at 30°C with excitation and emission wavelengths of 340 and 460 nm, respectively, in 50 mM sodium MES buffer (pH 6.0) containing 10 μM NADH and 1 to 4 μM wild-type and mutant D-LDHs. Concentration of NADH was determined by measuring fluorescence intensity of various concentrations of NADH at 30°C in 50 mM sodium MES buffer (pH 6.0).

RESULTS AND DISCUSSION

Effects of Replacement of Tyr52 with Aliphatic Amino Acids on the Catalytic Activity of L. pentosus *D-LDH*—Table 2 summarizes the kinetic parameters of the Y52V and Y52A mutant *L. pentosus* D-LDHs for various 2-ketoacid substrates, together with those of the wild-type and Y52L enzymes, and Fig. 3 shows apparent changes in the free energies of transition-state binding (ΔG) by these Tyr52 replacements. As in the case of the replacement of Tyr52 with Leu (*36*), replacement with Val or Ala greatly decreased the catalytic activity toward pyruvate and increased the activity toward larger aliphatic 2-ketoacid substrates such as 2-ketoisocaproate. However, the Y52V and Y52A enzymes exhibited lower k_{cat}/K_m toward 2-ketobutyrate than the wild-type enzyme, unlike the Y52L enzyme, and apparently required larger substrates for their efficient catalysis than the Y52L enzyme did. The Y52V enzyme generally exhibited lower catalytic activity toward aliphatic substrates than the Y52L enzyme (ΔG of 1 to 2 kcal/mol), and higher catalytic activity than the Y52A enzyme (also ΔG of 1 to 2 kcal/mol), except for pyruvate and 2-ketocaproate. These results indicate that the capacity of the substrate binding pocket depends greatly on the size of the side chain at position 52. For pyruvate and 2-ketocaproate, nevertheless, the Y52V and Y52A enzymes exhibited virtually the same k_{cat}/K_m values, which were 964- and 1,350-fold lower (ΔG = 4.1 and 4.3 kcal/mol) for pyruvate, and only 270- and 232-fold higher (ΔG = 3.4 and 3.3 kcal/mol) for 2-ketocaproate than those of the wild-type enzyme, respectively. It is imaginable that the binding pockets of the two enzymes are too wide to accommodate the C3 methyl group of pyruvate. Furthermore, the long aliphatic chain of 2-ketocaproate may extend out of the binding pocket, where its C6 methyl group may be able to make contact with the Leu side chain, but not with the smaller side chains of Val and Ala. On the other hand, the two enzymes exhibited no significant activity toward 2-ketoisovalerate, which is a poor substrate for both the wild-type and Y52L enzymes.

These Tyr52 replacements markedly changed the k_{cat} values for the reactions, besides the predominant changes in the K_m values (Table 2). For the pyruvate reduction, for example, the Y52V and Y52A enzymes exhibited 8.6- and 12.6-fold lower k_{ca} and 110- and 105-fold larger K_m than the wild-type enzyme, and 5.8- and 8.4-fold lower k_{ca} and 7.2- and 7.0-fold larger K_m than the Y52L enzyme, respectively (Table 2). Therefore, the Leu52 to Val and Ala replacements affected the k_{cat} and K_m values for pyruvate equally. Table 2 also shows the primary isotope effects, which correspond to the effects on both the k_{cat} and k_{cat}/K_m, since NADH and NADD give virtually the same K_m values (data not shown). In pyruvate reduction, the Y52A and Y52V enzymes, and even Y52L enzyme, which exhibits

Table 2. **Kinetic parameters for various substrates for Y52L, Y52V and Y52A *L. pentosus* D-LDHs.**

	Wild-type				Y52L				Y52V				Y52A			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D	K_m[a] (mM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D
Pyruvate	0.12	321	2,700	1.6	1.8	213	120	2.7	13.1	37	2.8	2.5	12.6	25.5	2.0	2.6
2-Ketobutyrate	8.1	118	15	2.6	0.38	175	460	2.5	3.5	41	12	2.2	20.8	41.5	2.0	2.4
2-Ketovalerate	17	5.7	0.34	1.9	0.15	334	2,200	2.2	0.8	102	128	2.1	11.7	55.7	4.8	2.8
2-Ketoisovalerate	27	5.5	0.2	2.9	11.7	18.6	1.6	3.3	ND[b]	ND	ND	ND	ND	ND	ND	ND
2-Ketocaproate	21	13.3	0.63	3.0	0.056	189	3,400	2.5	0.3	51	170	2.1	0.7	102	146	2.7
2-Ketoisocaproate	31	11.1	0.36	3.0	0.124	65.7	530	2.5	0.7	17	24	2.5	12.1	15.8	1.3	3.0
Phenylpyruvate	0.8	40	50	2.7	0.067	778	11,600	1.4	0.14	171	1,220	1.3	0.03	232	7,730	1.9
Hydroxypyruvate	0.28	257	920	2.9	3.0	407	140	2.0	8.7	50	5.7	2.0	17	104	6.1	2.7
Oxaloacetate	2.2	280	127	2.1	–[c]	–	–	–	–	–	–	–	–	–	–	–
2-Ketoglutarate	15.5	0.51	0.03	2.6	–	–	–	–	–	–	–	–	–	–	–	–

[a]Cited from Ref. *36*. [b]ND, not determined (enzyme activity was too weak for determination of exact values). [c]–, no data.

Fig. 3. **Apparent changes in free energy (Δ*G*) of transition-state binding for aliphatic and aromatic 2-ketoacid substrates by the Tyr52 to Leu (open boxes), Val (hatched boxes) and Ala (closed boxes) replacements.** Δ*G* were calculated from $\Delta G = RT\ln[(k_{cat}/K_m \text{ of the mutant enzyme})/(k_{cat}/K_m \text{ of the wild-type enzyme})]$, using the kinetic parameters listed in Table 2.

only slightly (1.5-fold) decreased k_{cat}, showed much higher isotope effects (2.5 to 2.7) than that of the wild-type enzyme (1.6), indicating that the hydrogen transfer step became a further rate-limiting step following these Tyr52 replacements. It is imaginable that Tyr52 allows the bound pyruvate molecule to suitably interact with the His296 imidazole or the NADH nicotinamide ring in the transition state, possibly through tightly fixing the C3 methyl group of pyruvate with its phenol side chain.

In the case of the wild-type enzyme, large aliphatic substrates gave larger isotope effects than pyruvate (Table 2), indicating that the hydrogen transfer step is fully rate-limiting, unlike the case of pyruvate. The Tyr52 replacements did not markedly reduced these isotope effects, but rather increased the effects for certain substrates such as 2-ketovalerate, in spite of the greatly increased k_{cat}. These results suggest that the Tyr 52 replacements enhance not only the hydrogen transfer step but also the other step(s) of the catalytic process. The active site of *L. pentosus* D-LDH is located in a deep cleft between the catalytic domain (positions 1 to 100 and 300 to 332) and the coenzyme binding domains (positions 101–299) in each of the two subunits (*25*). The apo and complex structures of the D-LDH and related enzymes (*6*, *7*, *25*, *26*, *35*) imply that the catalytic reaction of the enzyme involves marked conformational changes due to a flexible hinge motion of the catalytic domain, the catalytic cleft closing and opening when the enzyme binds and releases ligands, respectively. It is likely that such a protein motion is one of the slow steps in the catalytic reaction, and possibly stimulated by the binding of suitable substrate to the enzyme.

For phenylpyruvate reduction, these Tyr52 replacements not only greatly improve both the k_{cat} and K_m values, but also markedly reduced the isotope effects (Table 2). It was also notable that the Y52A enzyme exhibited 6-fold higher k_{cat}/K_m value than the Y52V enzyme (only 1.5-fold lower than the case of the Y52L enzyme), unlike in the case of aliphatic substrates, showing much higher preference toward phenylpyruvate than the other 2-ketoacid substrates. The benzene ring of phenylpyruvate appears to fit well into the binding pocket of the Y52A

enzyme through compensating for the missing Tyr52 phenol. The Y52A enzyme exhibited a slightly larger isotope effect than the Y52L and Y52V enzyme and a predominantly improved K_m value for phenylpyruvate, while the Y52L and Y52V enzymes gave equally improved k_{ca} and K_m values. These results suggest that the replacement of Tyr52 with aliphatic amino acids improves various catalytic steps in the phenylpyruvate reduction, depending on the size or shape of the aliphatic side chain.

Effects of Replacement of Tyr52 with Arg, Thr and Asp, and of Phe299 with Gly and Ser on the Catalytic Activity of L. pentosus *D-LDH*—The replacement of Tyr52 with Arg, Thr or Asp induced a great reduction of the enzyme activity toward all the substrates listed in Table 3. Among these mutant enzymes, only the Y52R enzyme exhibited weak but significant catalytic activity toward certain 2-ketoacid substrates (Table 3). This enzyme exhibited no detectable activity toward small aliphatic 2-ketoacids such as pyruvate and 2-ketobutyrate, but significant activity toward larger substrates such as 2-ketovalerate, 2-ketocaproate and phenylpyruvate, giving 10^{-3} to 10^{-1}-fold lower k_{cat}/K_m values than the wild-type enzyme. This result indicates that the replacement of Tyr52 with Arg induces a similar substrate preference as do the replacements with aliphatic amino acids, in spite of the great reduction of the enzyme activity. It is imaginable that the aliphatic moiety of Arg provides the enzyme with such a substrate preference, though the guanidium moiety hinders the enzyme activity. The Y52R enzyme also weakly catalyzed the reductions of hydroxypyruvate and oxaloacetate, which were favorable substrates for the wild-type (Table 2), with greatly reduced k_{cat}/K_m (Table 3).

On the other hand, the replacement of Phe299 with Gly or Ala also greatly reduced the activity toward all the substrates tested (Table 3). Unlike the Y52R enzyme, however, both the F299G and F299S enzymes exhibited no detectable activity toward 2-ketovalerate or 2-ketocaproate, but significant activity toward pyruvate with about 10^{-4}-fold decreased k_{cat}/K_m. The two enzymes also significantly catalyzed the reduction of phenylpyruvate and hydroxypyruvate with 10^{-2} to 10^{-3}-fold reduced catalytic efficiency. Consequently, the two enzymes only exhibited significant activity toward the favorable substrates for the wild-type enzyme, showing a similar substrate preference to the wild-type enzyme, unlike the case of the Y52R enzyme. It was, however, notable that the F299S enzyme exhibited markedly higher activity toward hydroxypyruvate and oxaloacetate than the F299G enzyme, unlike the cases of the other substrates, to which the two enzymes had essentially the same catalytic activity. This implies that the side chain at position 299 can participate in the substrate recognition at least for the case of the 2-ketoacid substrates that have hydrophilic side chains. The preference of the F299S enzyme for hydroxypyruvate is consistent with the fact that cucumber (*13*) and *H. methylovorum* (*14*) D-glycerate dehydrogenases (hydroxypyruvate reductase) possess a Ser at the position corresponding to Phe299 (Fig. 2).

Table 4 summarizes the kinetic parameters of the Y52R/F299G and Y52T/F299S double mutant enzymes, which have Arg52 and Gly299, and Thr52 and Ser299 instead of Tyr52 and Phe299, respectively. The Y52R/F299G enzyme exhibited no detectable activity toward pyruvate, 2-ketovalerate, or oxaloacetate. These negative

Table 3. **Kinetic parameters for various substrates for Y52R, F299G and F299S *L. pentosus* D-LDHs.**[a]

	Y52R				F299G				F299S			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D
Pyruvate	ND[b]	ND	ND	ND	31 (260)	22 (6.9×10^{-2})	0.71 (2.6×10^{-4})	2.5	>50	ND	0.49 (1.8×10^{-4})	2.4
2-Ketobutyrate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketovalerate	ND	ND	0.02 (5.9×10^{-2})	1.4	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketoisovalerate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketocaproate	15.9 (0.76)	1.18 (9×10^{-2})	0.074 (1.2×10^{-1})	2.6	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketoisocaproate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phenylpyruvate	2.1 (2.6)	0.31(7.8×10^{-3})	0.15 (3.0×10^{-3})	1.8	22 (28)	3.4 (8.5×10^{-2})	0.15 (3.0×10^{-3})	2.9	7.2	2.1 (5.3×10^{-2})	0.29 (5.8×10^{-3})	2.6
Hydroxypyruvate	20.2 (72)	13.8 (5.4×10^{-2})	0.68 (7.4×10^{-4})	1.4	53 (190)	8.1 (3.2×10^{-2})	0.15 (1.6×10^{-4})	2.5	18.6 (66)	35.8 (1.4×10^{-1})	1.9 (2.1×10^{-3})	2.7
Oxaloacetate	3.8 (1.7)	0.48 (1.7×10^{-3})	0.13 (1.0×10^{-3})	1.8	ND	ND	ND	ND	43.1 (20)	6.5 (2.3×10^{-2})	0.15 (1.2×10^{-3})	2.3
2-Ketoglutarate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]The values in parentheses are those relative to the wild-type enzyme. [b]ND, not detectable (enzyme activity was too weak for determination of exact values).

Table 4. **Kinetic parameters for various substrates for Y52R/F299G and Y52T/F299S *L. pentosus* D-LDHs.**

	Y52R/F299G				Y52T/F299S			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_H/k_D
Pyruvate	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketobutyrate	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketovalerate	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketoisovalerate	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketocaproate	14.1 (14)	0.36 (2.7×10^{-2})	0.026 (4.1×10^{-2})	2.4	ND	ND	ND	ND
2-Ketoisocaproate	ND	ND	ND	ND	ND	ND	ND	ND
Phenylpyruvate	6.9 (8.6)	2.8 (7.0×10^{-2})	0.41 (8.2×10^{-3})	1.9	8.7 (11)	5.2 (1.3×10^{-1})	0.6 (1.2×10^{-2})	2.5
Hydroxypyruvate	>50(>180)	ND	0.012 (1.3×10^{-5})	1.4	19 (68)	2.1 (8.2×10^{-3})	0.11 (1.2×10^{-4})	1.7
Oxaloacetate	ND	ND	ND	ND	ND	ND	ND	ND
2-Ketoglutarate	8.7 (0.56)	0.89 (1.7)	0.1 (3.3)	2.7	ND	ND	ND	ND

[a]The values in parentheses are those relative to the wild-type enzyme. [b]ND, not determined (enzyme activity was too weak for determination of exact values).

characteristics are consistent with those of the Y52R and P299G single mutant enzymes. Nevertheless, this enzyme catalyzed 2-ketocaproate reduction with only slightly lower catalytic efficiency than the Y52R enzyme, while the F299G enzyme did not significantly catalyze this reaction. In addition, this enzyme exhibited only 1.3×10^{-5}-fold lower activity toward hydroxypyruvate than the wild-type enzyme, while the Y52R and F299G single mutant enzymes showed 6.5×10^{-5} and 1.6×10^{-4}-fold reduced activities. These results indicate that the mutations Y52R and F299G do not synergically or additionally reduce the enzyme activity toward these substrates. It was particularly notable that the Y52R/F299G enzyme exhibited a higher activity toward 2-ketoglutarate even than the wild-type enzyme, while the two single mutant enzymes were virtually inert to 2-ketoglutarate. This result demonstrates that the two mutations mutually suppress their negative effects on the 2-ketoglutarate reduction, and induce a new preference for 2-ketoglutarate in the enzyme. This substrate preference of the Y52R/F299G enzyme is in good agreement with the fact that 2-ketoglutarate is one of the favorable substrates for *E. coli* D-PGDH (*42*, *43*), which possesses an Arg and Gly at the positions of Tyr52 and Phe299, respectively (Fig. 2). On the other hand, the Y52R/F299G enzyme exhibited no apparent activity toward oxaloacetate, unlike the wild-type or Y52R single mutant enzyme. It is imaginable that the aliphatic chain of oxaloacetate is too short to statically interact with the Arg guanidino group. The wild-type and Y52R single mutant enzymes, which catalyzed oxaloacetate reduction, appear to bind the oxaloacetate side chain by other means than the static interaction.

The Y52T/F299S double mutant enzyme exhibited significant activity toward phenylpyruvate and hydroxypyruvate (Table 4), while the Y52T single mutant enzyme was virtually inert to any tested substrates including these two. This result indicates that the Phe299 to Ser replacement also suppresses the negative effects of the Tyr52 to Thr replacement on the reactions for these two substrates. On the other hand, the Y52T/F299S enzyme did not exhibit significant activity toward pyruvate or oxaloacetate, to which the F299S single mutant enzyme exhibited weak but significant activity, and consequently showed more strict substrate preference for phenylpyruvate and hydroxypyruvate than the F299S enzyme. The preference of the F299S enzyme for hydroxypyruvate is again consistent in the fact that *H. methylovorum* D-glycerate dehydrogenases have a Thr for Tyr52 (Fig. 2).

Table 5. **Maximal changes in fluorescence intensity (ΔF) and dissociation constants (K_d) of NADH, and rates of NADH oxidation under substrate-free conditions by the wild-type and mutant *L. pentosus* D-LDHs.**

	Relative ΔF	K_d (μM)	Rate constant (k) of NADH oxidation (s^{-1})
Wild-type	1	3.4	ND[a]
Y52L	0.91	1.4	ND
Y52V	0.93	2.4	ND
Y52A	0.73	4.9	ND
Y52R	0.22	–[b]	5.7×10^{-3}
Y52T	ND	–	8.0×10^{-3}
Y52D	0.19	–	5.2×10^{-3}
F299G	ND	–	1.6×10^{-3}
F299S	0.78	1.5	ND
Y52R/F299G	0.23	1.6	ND
Y52T/F299S	0.20	1.8	ND

[a]ND, not determined (the value is in control level of enzyme-free conditions). [b]–, no data.

The amino acid side chains at positions 52 and 299 are near enough to interact with each other as well as the side chain of substrate (Fig. 1). It is, therefore, likely that the charged or polar group at position 52 and the Phe299 benzene ring repulse and perturb each other. The replacement of Phe299 with Gly or Ser must relieve such a perturbation, and thereby reconstruct the recgnition pocket for 2-ketoacid substrates. In the case of the Y52R enzyme, the Phe299 benzene ring may hinder the guanidino group of Arg52 from forming static interactions with the carboxyl group of the 2-ketoglutarate side chain, but this is removed by the further replacement of Phe299 with Gly. Thus, the results for the double mutant enzymes indicate that the cooperative and coodinative combination of the two amino acid residues is pivotal in the recognition or preference of the enzyme for the 2-ketoacid side chains.

Effects of Replacements of Tyr52 and Phe299 on the NADH Binding of L. pentosus *D-LDH*—Table 5 summarizes the data on the NADH binding of the wild-type

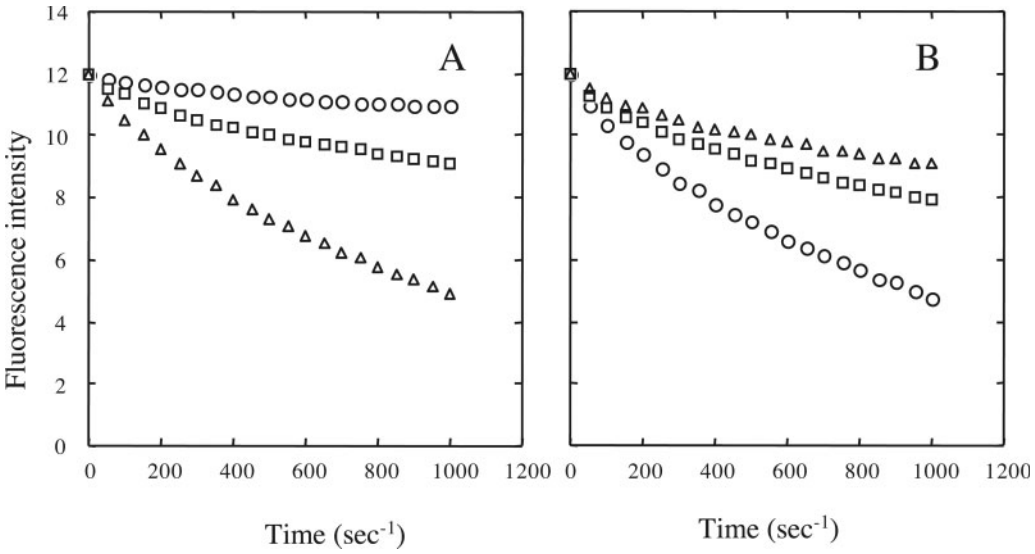

Fig. 4. **2-Ketoacid substrate-independent NADH oxidation by F299G mutant *L. pentosus* D-LDHs.** Panel A: Dependence of the oxidation rate on the enzyme concentration. The reaction mixtures contained 10 μM NADH and 1 (circles), 2 (squares) and 4 (triangles) μM enzyme. Panel B: Protection of NADH from the oxidation by oxamate. The reaction mixtures conained 10 μM NADH, 4 μM the enzyme, and 0 (circles), 10 (squares) and 100 (triangles) mM sodium oxamate.

and mutant enzymes, gained by NADH fluorescence analysis. All the mutant enzymes exhibited significantly lower change of NADH fluorescence (ΔF), and the Y52T and F299G enzymes showed no significant ΔF. The exact dissociation constants (K_d) of NADH could not be determined for some enzymes which exhibited significant oxidation of NADH during the fluorescence measurement, as described below. The other enzymes exhibited significantly smaller NADH K_d than the wild-type enzyme, except for the Y52A enzyme, which showed slightly larger NADH K_d. These results indicate that the replacement of Tyr52 or Phe299 significantly affects the affinity of the enzyme for NADH, and the environment surrounding the nicotinamide moiety of the bound NADH molecule. This is not unreasonable, since Tyr52, Phe299 and the NADH nicotinamide are located near one another (Fig. 1).

It was notable that the Y52R, Y52T, Y52D and F299G enzymes significantly oxidized NADH in the absence of 2-ketoacid substrates, during monitoring the fluorescence intensity. This substrate-independent oxidation of NADH was significantly faster than the background level and clearly depended on the concentration of the enzyme, and then displayed a saturation profile for NADH concentration. For the representative case, Fig. 4 shows the data for the F299G enzyme, which did not exhibit significant ΔF (Table 5). The substrate-independent NADH oxidation was slow enough to be negligible for the determination of the enzyme activity toward 2-ketoacids, but not for the determination of NADH K_d values for these mutant enzymes. Since the oxidation was sufficiently saturated below 4 μM of NADH (versus 1 μM of the enzymes) (data not shown), nevertheless, it is likely that these enzymes can bind NADH strongly as the other enzymes. Table 5 also shows the rate constants (k) of the NADH oxidation by these enzymes under saturation level of NADH (10 μM).

The NADH oxidation by the F299G enzyme was markedly inhibited in the presence of oxamate (Fig. 4, panel B), which is an inert pyruvate analogue and inhibits the enzyme reaction competitively toward substrate (*1*). The F299G enzyme exhibited a much larger inhibition constant (80 mM) for oxamate than the wild-type enzyme (2.8 mM) (*23*, *27*), being consistent in the larger pyruvate K_m of the F299G enzyme (31 mM) than that of the wild-type enzyme (0.12 mM). This inhibitory effect of oxamate appeared to be also consistent in its effect on NADH protection in the F299G enzyme, since the oxidation was half-inhibited by 10 to 100 mM oxamate (Fig. 4, panel B). Since the carbonyl group of oxamate interacts with the reactive C4 hydrogen (hydride) of the NADH nicotinamide, as in the case of substrate pyruvate (*1*), it is reasonable that oxamate protects the C4 hydride from the solvent if the F299G enzyme correctly binds NADH and oxamate like the wild-type enzyme.

The substrate-independent NADH oxidation implies that the nicotinamide of bound NADH, which may be more reactive than that of free NADH, is highly exposed to the solvent in these mutant enzymes. This is reasonable for the F299G enzyme, since the benzene ring of Phe299 is positioned in the active site so as to cover and shield the nicotinamide from the solvent (Fig. 1). In addition, Tyr52 is located near Phe299, and its replacement with a charged or polar amino acid such as Arg, Asp or Thr may perturb the position or orientation of the Phe299 side chain. Interestingly, neither the Y52R/F299G nor the Y52T/F299S enzyme exhibited such a substrate-independent NADH oxidation, unlike each of the single mutant enzymes (Table 5), indicating that Arg52 and Gly299, and Thr52 and Ser299 constitute alternative combinations that efficiently protect NADH from the solvent. The long side chain of Arg52 may fulfill the role of Phe299 for the NADH protection, and the hydrophilic side chain of Thr does not perturb that of Ser299. Thus, these results strongly suggest that the suitable combination of the two side chains produces an efficient shield for NADH from the solvent. Such a shield may also stimulate the substrate binding or hydride transfer through isolating the nicotinamide or substrate from the solvent.

Conclusion—The results in this study lead to the following conclusions. 1. The cooperative and coordinative combination of Tyr52 and Phe299 provides *L. pentosus* D-LDH not only with the high activity and preference for pyruvate, but also the sufficient shield for the reactive NADH from the solvent. 2. Replacement of Tyr52 with aliphatic amino acids such as Leu, Val and Ala produces alternative

combinations with Phe299 that provide the enzyme with high activity and preference for longer aliphatic 2-ketoacid substrates and phenylpyruvate, through stimulating various steps of the catalysis for these substrates. 3. The preference for each of these substrates depends on the size and shape of the chains of the substitutive amino acids. 4. The replacement of Tyr52 with a polar or charged amino acid such as Thr or Arg requires additional replacement of Phe299 to construct another suitable combination that protects the bound NADH and provides the enzyme with a new preference for substrates that have a polar or charged group on the side chain. These results thus strongly suggest that the multiple combinations of the two amino acid residues are pivotal for the great variety in the physiological and catalytic functions of the D-2-HydDH superfamily.

This work was supported by a Grant-in-Aid for Science Research to H. T. from the Ministry of Education, Science, and Culture of Japan.

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