Regulation of the Activity of Lactate Dehydrogenases from Four Lactic Acid Bacteria

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Background: Lactate dehydrogenases (LDHs) are key metabolic enzymes in lactic acid bacteria (LAB).
Results: The effects of fructose 1,6-bisphosphate, phosphate, pH, and ionic strength on enzyme activity differ for six LDHs from four LAB.
Conclusion: The regulation of LDH activity differs among LAB.
Significance: These results have implications for understanding enzyme evolutionary adaptation, for quantitative comparative modeling, and for biotechnological application of LAB.

Despite high similarity in sequence and catalytic properties, the l-lactate dehydrogenases (LDHs) in lactic acid bacteria (LAB) display differences in their regulation that may arise from their adaptation to different habitats. We combined experimental and computational approaches to investigate the effects of fructose 1,6-bisphosphate (FBP), phosphate (P_i), and ionic strength (NaCl concentration) on six LDHs from four LABs studied at pH 6 and pH 7. We found that 1) the extent of activation by FBP (K_aFBP) differs. Lactobacillus plantarum LDH is not regulated by FBP, but the other LDHs are activated with increasing sensitivity in the following order: Enterococcus faecalis LDH2 < Lactococcus lactis LDH2 < E. faecalis LDH1 < L. lactis LDH1 < Streptococcus pyogenes LDH. This trend reflects the electrostatic properties in the allosteric binding site of the LDH enzymes. 2) For L. plantarum, S. pyogenes, and E. faecalis, the effects of P_i are distinguishable from the effect of changing ionic strength by adding NaCl. 3) Addition of P_i inhibits E. faecalis LDH2, whereas in the absence of FBP, P_i is an activator of S. pyogenes LDH, E. faecalis LDH1, and L. lactis LDH1 and LDH2 at pH 6. These effects can be interpreted by considering the computed binding affinities of P_i to the catalytic and allosteric binding sites of the enzymes modeled in prototype states corresponding to pH 6 and pH 7. Overall, the results show a subtle interplay among the effects of P_i, FBP, and pH that results in different regulatory effects on the LDHs of different LABs.

Lactobacillus plantarum, Streptococcus pyogenes, Enterococcus faecalis, and Lactococcus lactis are Gram-positive microorganisms belonging to the phylogenetic order Lactobacillales. These bacteria have their natural habitats in rather different environments (L. lactis, milk; L. plantarum, plants; E. faecalis, feces; S. pyogenes, skin and mucosal membranes) and interact differently with human beings. L. lactis and L. plantarum are bacteria of major importance for use in the food industry. E. faecalis is an important commensal of the human gut, a food contaminant, and a facultative pathogen. S. pyogenes is an exclusively human pathogen causing diseases like tonsillitis, pharyngitis, scarlet fever, and necrotizing fasciitis (1–5). Despite their different lifestyles, all four species predominantly gain energy by homolactic acid fermentation. The free energy generated during homolactic acid fermentation is 2 mol of ATP/1 mol of glucose. The crucial enzyme in this pathway is LDH, which is responsible for catalyzing the reversible reduction of pyruvate to lactate. This reaction serves solely to balance the redox potential by oxidation of NADH + H+ to NAD+

The LAB genomes differ in the number and type of LDH enzymes that they encode. The L. plantarum strain WCFS1 genome encodes at least two LDHs catalyzing the production of l-lactate (lp_0537, l-LDH1 and lp_1101, l-LDH2) and one producing d-lactate (lp_2057, d-LDH) (6). (The LDH locus and

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corresponding protein short names as they appear in the UniProt database are given in parentheses.) The genome of the S. pyogenes M49 strain NZ131 encodes for one \( \alpha \)-LDH (spy49_0904c, \( \alpha \)-LDH) and one \( \delta \)-LDH (spy49_0919, \( \delta \)-LDH) (7, 8). In the E. faecalis strain V583 genome, two \( \lambda \)-lactate dehydrogenases (ef_0255, \( \lambda \)-LDH1 and ef_0641, \( \lambda \)-LDH2) are encoded (9, 10). The E. faecalis genome additionally encodes a \( \beta \)-LDH involved in vancomycin resistance by incorporation of \( \beta \)-lactate instead of \( \alpha \)-alanine in the cell wall (9, 11). The L. lactis strain MG1363 genome possesses three \( \alpha \)-LDH genes (llmg_1120, \( \alpha \)-LDH1; llmg_0392, \( \alpha \)-LDH2; and llmg_1429, \( \lambda \)-LDH) (12). This study is restricted to \( \alpha \)-LDH enzymes, which we will refer to simply as LDH.

The activation of \( \alpha \)-LDHs extracted from L. lactis and E. faecalis was studied by Crow and Pritchard (13), but they did not distinguish between the two types of enzyme in each organism. Gaspar et al. (14) later characterized the kinetics of the individual L. lactis LDH1 and LDH2 isoenzymes. However, knowledge of the kinetics and allosteric regulation of the respective isoenzymes from L. plantarum, S. pyogenes, and E. faecalis remains lacking.

The crystal structure of a bacterial LDH is shown in Fig. 1. The biological assembly is a homotetramer. Each monomer has one active site, and the tetramer has two allosteric sites, each situated at the interface between two monomers. Fructose 1,6-bisphosphate (FBP) has been shown to allosterically regulate some LDHs, including L. lactis LDHs, and to bind at these allosteric sites. The mechanism of LDH regulation by FBP has been defined to be allosteric because of a sequential intersubunit rearrangement of the LDH tetramer accompanied by local intrasubunit conformational changes (15, 16). However, it has also been shown that the L. plantarum LDH1 is not allosterically regulated, and this has been ascribed to the presence of an aspartic acid residue in the allosteric site (17).

To provide a detailed understanding of the kinetics and regulation of LAB LDHs, we undertook a study combining both experimental and computational approaches. We determined the kinetic parameters and the allosteric regulation of the heterologously expressed LDH isoenzymes from L. plantarum, S. pyogenes, and E. faecalis at pH 6 and pH 7 in the presence of varying concentrations of FBP, inorganic phosphate (P\(_i\)), and sodium chloride (NaCl). Structural models of the LDH enzymes and comparative computational analyses of their binding properties were used to interpret the experimental data. This analysis allowed us to propose a unified model of the regulatory mechanisms of LAB LDHs.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—Escherichia coli DH5\( \alpha \) strains harboring recombinant plasmid DNA were cultivated in lysogeny broth (LB) medium containing 100 mg/liter ampicillin at 37 °C in shaking cultures. S. pyogenes M49 591, E. faecalis V583, and L. plantarum WCFS1 strains were grown in Todd Hewitt broth supplemented with 0.5% yeast extract or brain-heart infusion medium at 37 °C as standing cultures.

**Construction of Recombinant Plasmids**—The chromosomal DNA of E. faecalis V583 and L. plantarum WCFS1 was isolated according to the Qiagen Blood and Tissue kit (Qiagen, Hilden, Germany) and used as the template for PCR amplification of the ldh genes with the Phusion\textsuperscript{TM} High Fidelity PCR kit (Finnzymes). The resulting PCR fragments were ligated into the pASK-IBA vector (IBA GmbH, Göttingen, Germany) system via BamHI and SalI restriction sites. The recombinant vectors were transformed in E. coli DH5\( \alpha \) cells. Correct insertion of the PCR products was confirmed by plasmid sequencing. The construction of the expression plasmid for the S. pyogenes M49 591 ldh gene has been described previously (18).

**Expression and Purification of Proteins**—For heterologous expression of the isoenzymes, recombinant E. coli strains were grown in 500 ml of LB medium at 37 °C under vigorous shaking. At an optical density of about 0.4, expression was induced by addition of anhydrotetracycline (0.2 \( \mu \)g/ml). Cells were harvested after overnight shaking at 22 °C, and pellets were stored at –80 °C. For purification of the Strep-tagged proteins, cell pellets (from 500 ml of culture) were thawed and suspended in 8 ml of buffer W (100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl), and cell disruption was achieved by the FastPrep method with acid-washed glass beads. Five cycles of 30 s each at a speed of 6.0 m\textsuperscript{s}–\textsuperscript{1} were applied. In between, samples were cooled on ice for at least 2 min. Cell debris was removed by centrifugation. Clear supernatants were loaded on Strep-Tactin-Sepharose columns (5 ml volume) prewashed with buffer W (3 times column volume). Unbound proteins were washed from the column with buffer W (5–7 times column volume). The protein carrying a Strep tag was eluted from the column in fractions of 0.5 ml each in buffer E. Buffer E is buffer W supplemented with 2.5 mM desthiobiotin. Elution fractions were checked for purified protein with SDS-PAGE. Fractions containing pure protein were pooled and dialyzed overnight in 100 mM MES buffer to prepare them for enzymatic assays.

**Protein Concentration Measurements**—The protein concentrations in the purified recombinant isoenzyme fractions were determined by the Bradford method using the Bio-Rad Protein Assay kit.

**Enzymatic Assays**—The LDH activity was determined in the forward and reverse reactions by measuring the rate of NADH oxidation for the forward reaction and the rate of NAD reduction for the reverse reaction at 380 nm using an extinction coefficient for NADH of 1.244 mm\textsuperscript{–1} cm\textsuperscript{–1}. All assays were carried out at 30 °C in 60-\( \mu \)l reaction volumes in 384-well format plates, and the time course of NADH absorbance was monitored with a BMGLabtech NOVOStar plate reader (automated fluorescence/fluorescence polarization/absorbance reader; BMGLabtech, Offenburg, Germany). The assays were automated so that all the reagents in the reaction buffer were in 45 \( \mu \)l, the enzyme was in 5 \( \mu \)l, and the substrate was in 10 \( \mu \)l. The assay setup was similar to that used previously (19). Full sets of experiments could be run simultaneously for each isoenzyme with its two substrates and at two pH values (pH 6 and pH 7) for the forward and reverse reactions, allowing quick production and reliable comparison of data. For measuring the activity of LDH, a standard assay mixture of 100 mM MES/KOH, pH 7.5, 5 mM MgCl\textsubscript{2}, 3 mM FBP, 1 mM NADH was used and incubated with a suitable concentration of the enzyme at 30 °C. Then 10 mM pyruvate was added to start the reaction. For determining the kinetic parameters of the different isoenzymes

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These extracted sections from the text provide a detailed overview of the regulation of lactate dehydrogenase activity, focusing on the experimental procedures and the analysis of LAB LDHs. The text delves into the methodologies used for expressing and purifying these enzymes, as well as the assays employed to measure their activity. The study includes a detailed description of the expression and purification of proteins for these isoenzymes, followed by enzymatic assays to determine the kinetic parameters and allosteric regulation of the enzymes. This comprehensive approach aims to provide a unified model of the regulatory mechanisms of LAB LDHs.
at pH 6 and pH 7 for the forward and reverse reactions and the effect of allosteric activators, the following measurements were conducted: 1) the kinetic parameters in the absence and presence of FBP at a fixed concentration of NADH/NAD using various concentrations of pyruvate/lactate for the $K_m$ of pyruvate/lactate and at a fixed concentration of pyruvate/lactate using various concentrations of NADH/NAD for the $K_m$ of NADH/NAD and 2) the effect of FBP, $P_i$, and ionic strength (NaCl) on the LDH activity by conducting assays at substrate concentrations equal to the $K_m$ values and not at saturated concentrations. $K_{act}$ values were determined whenever an allosteric activation was found by measuring the activity of the enzyme at various concentrations of the activator. The effect of activators was also checked in the absence/presence of other activators. To determine whether or not the activation by $P_i$ is solely due to the change in ionic strength, measurements were made with different concentrations of NaCl.

All measurements were based on at least duplicate determinations of the reaction rates, and for all assays, control experiments were run in parallel to check and correct for any unwanted background activity. The data obtained were analyzed with KineticsWizard software (20). Data fitting was performed with the default settings according to the Michaelis-Menten equation.

**Comparative Modeling**—Modeling of the structures of the LDHs from *L. plantarum* (UniProt identifier P56512), *S. pyogenes* (P56259), *E. faecalis* (Q839C1 and Q838C9 for LDH1 and LDH2, respectively), and *L. lactis* (Q01462 and Q9CII4 for LDH1 and LDH2, respectively) was performed with MODELLER8v.2 (21).

The crystal structure of the LDH from *Bacillus steatorrhophilus* (Protein Data Bank code 1LDN) determined (22) at 2.5-Å resolution and shown in Fig. 1 was used as a template. The sequence alignment of the target LDHs shows a high similarity to the target LDHs.

**TABLE 1**

| Kinetic parameters | 0 mM FBP | 3 mM FBP |
|--------------------|----------|----------|
|                    | pH 6     | pH 7     | pH 6     | pH 7     |
| $L. plantarum$ LDH1 |          |          |          |          |
| $k_{cat}\text{forward}$ ($s^{-1}$) | $2.7 \pm 1.6\%$ | — | $n/a$ | $n/a$ |
| $K_{m\text{pyruvato}}$ (mM) | $4.3 \pm 10.2\%$ | — | $n/a$ | $n/a$ |
| $K_{m\text{NADH}}$ (mM) | $0.8 \pm 6.4\%$ | — | $n/a$ | $n/a$ |
| $k_{cat}\text{reverse}$ ($s^{-1}$) | $0.7 \pm 2.5\%$ | — | $n/a$ | $n/a$ |
| $K_{m\text{lactate}}$ (mM) | $103.0 \pm 7.7\%$ | — | $n/a$ | $n/a$ |
| $K_{m\text{NAD}}$ (mM) | $n/a$ | $n/a$ | $n/a$ | $n/a$ |
| $S. pyogenes$ LDH |          |          |          |          |
| $k_{cat}\text{forward}$ ($s^{-1}$) | Very slow | Very slow | $35.9 \pm 2.0\%$ | $31.3 \pm 2.1\%$ |
| $K_{m\text{pyruvato}}$ (mM) | — | — | $1.7 \pm 7.6\%$ | $1.7 \pm 5.5\%$ |
| $K_{m\text{NADH}}$ (mM) | — | — | $0.6 \pm 5.7\%$ | $0.6 \pm 6.2\%$ |
| $k_{cat}\text{reverse}$ ($s^{-1}$) | Very slow | Very slow | $2.6 \pm 2.5\%$ | $9.2 \pm 1.6\%$ |
| $K_{m\text{lactate}}$ (mM) | — | — | $112.0 \pm 7.6\%$ | $98.0 \pm 5.2\%$ |
| $K_{m\text{NAD}}$ (mM) | — | — | $2.3 \pm 7.0\%$ | $2.3 \pm 3.8\%$ |
| $E. faecalis$ LDH1 |          |          |          |          |
| $k_{cat}\text{forward}$ ($s^{-1}$) | $4.5 \pm 2.2\%$ | $4.3 \pm 1.9\%$ | $20.4 \pm 2.4\%$ | $21.3 \pm 1.8\%$ |
| $K_{m\text{pyruvato}}$ (mM) | — | — | $2.7 \pm 6.6\%$ | $2.6 \pm 6.0\%$ |
| $K_{m\text{NADH}}$ (mM) | $1.6 \pm 4.9\%$ | $1.6 \pm 4.1\%$ | $0.5 \pm 7.4\%$ | $0.6 \pm 5.3\%$ |
| $k_{cat}\text{reverse}$ ($s^{-1}$) | Very slow | Very slow | $0.7 \pm 3.4\%$ | $3.2 \pm 3.5\%$ |
| $K_{m\text{lactate}}$ (mM) | — | — | $2.7 \pm 9.1\%$ | $2.7 \pm 9.4\%$ |
| $L. lactis$ LDH1* |          |          |          |          |
| $k_{cat}\text{forward}$ ($s^{-1}$) | — | — | $1.5 \pm 13.3\%$ | $1.7 \pm 11.7\%$ |
| $K_{m\text{pyruvato}}$ (mM) | — | — | $0.05 \pm 5.5\%$ | $0.06 \pm 10.3\%$ |
| $K_{m\text{NADH}}$ (mM) | — | — | — | — |
| $k_{cat}\text{reverse}$ ($s^{-1}$) | — | — | — | — |
| $K_{m\text{lactate}}$ (mM) | — | — | — | — |
| $K_{m\text{NAD}}$ (mM) | — | — | — | — |
| $L. lactis$ LDH2* |          |          |          |          |
| $k_{cat}\text{forward}$ ($s^{-1}$) | — | — | $1.3 \pm 7.8\%$ | $2.9 \pm 10.3\%$ |
| $K_{m\text{pyruvato}}$ (mM) | — | — | $0.08 \pm 2.6\%$ | $0.4 \pm 2.2\%$ |
| $K_{m\text{NADH}}$ (mM) | — | — | — | — |
| $k_{cat}\text{reverse}$ ($s^{-1}$) | — | — | — | — |
| $K_{m\text{lactate}}$ (mM) | — | — | — | — |
| $K_{m\text{NAD}}$ (mM) | — | — | — | — |
| $E. faecalis$ LDH2 |          |          |          |          |
| $k_{cat}\text{forward}$ ($s^{-1}$) | $4.1 \pm 3.2\%$ | $2.5 \pm 2.8\%$ | $13.3 \pm 5.3\%$ | $6.7 \pm 5.6\%$ |
| $K_{m\text{pyruvato}}$ (mM) | — | — | $24.3 \pm 9.4\%$ | $23.3 \pm 17.9\%$ |
| $K_{m\text{NADH}}$ (mM) | — | — | $4.3 \pm 15.1\%$ | $3.9 \pm 17.6\%$ |
| $k_{cat}\text{reverse}$ ($s^{-1}$) | — | — | $N/A$ | $N/A$ |
| $K_{m\text{lactate}}$ (mM) | — | — | $38 \pm 200$ | $38 \pm 200$ |
| $K_{m\text{NAD}}$ (mM) | — | — | $10.2 \pm 1.2\%$ | $13.3 \pm 1.3\%$ |

* Data from Ref. 14.

Although the selected Q01462 sequence for modeling *L. lactis* LDH1 is from strain IL1403, it shows 100% identity to the LDH1 from *L. lactis* strain MG1363. The sequence Q9CII4 (strain IL1403) for modeling *L. lactis* LDH2 has 97% identity to the LDH2 from *L. lactis* strain MG1363; there are no differences in charged residues within 10 Å of the FBP or the pyruvate binding sites.
Regulation of Lactate Dehydrogenase Activity

![Crystal structure of LDH from B. stearothermophilus](image)

**FIGURE 1. The crystal structure of the LDH from B. stearothermophilus** (22). The homotetrameric quaternary structure and the homodimeric structure, which was used as a template for modeling the LAB LDH structures, are shown in A and B, respectively. The catalytic and allosteric binding sites are indicated. The axes along the allosteric sites and the active sites are defined as P and Q axes, respectively (31). FBP molecules are shown in sphere representation in the allosteric binding sites. NAD, shown in orange; and oxamate (a pyruvate analog), shown in blue, are bound in the catalytic binding sites.

-ilarity of approximately 70% to the template (supplemental Fig. S1). Although the 1LDN structure is a homotetramer, only two monomers with an interfacial allosteric binding site for FBP were selected for the template. This homodimer contains one complete allosteric site and two complete active sites. Thus, for the purposes of this study, all LDHs were modeled in their homodimeric form. To ensure that differences in the modeled structures stemmed from their sequence only, we applied the protocol described by Gabdoulline et al. (23). This protocol uses the "automodel.very.fast" settings of MODELLER8v.2 and differs from the default procedure by not randomizing the initial coordinates and by performing a maximum of 50 steps of energy minimization without molecular dynamics refinement. After modeling, the LDH structures were resuperimposed on the template.

**Computation of Protein Electrostatic Potentials**—Polar hydrogen atoms were added at pH 7 to the protein structures by optimizing the hydrogen bond network with the WHAT IF 5.0 program (24). Electrostatic potentials were computed with the University of Houston Brownian Dynamics program (25). Partial atomic charges and atomic radii were assigned from the optimized potential for liquid simulations (OPLS) parameter set (26). Relative dielectric constants of 2 and 78 were assigned to the protein interior and solvent, respectively. Potentials were computed at an ionic strength of 150 mM on a cubic grid of 110 Å points with a 1-Å spacing. To obtain the electrostatic potential at pH 6, His-171 in the allosteric binding site and His-178 in the catalytic binding site were doubly protonated in all proteins at pH 6, His-171 in the allosteric binding site and His-178 in the catalytic binding site (CS) of LDH from L. plantarum were doubly protonated as residue 171 is an aspartic acid. Here, we use the sequence numbering of LDH1 for all LABs (supplemental Fig. S1).

**Comparison of the Allosteric Binding Sites**—The Protein Interaction Property Similarity Analysis (PIPSA) tool allows comparison of protein interaction fields (in this work electrostatic potentials) in the intersection of the "skins" of two superimposed structures (27). The "skin" region is defined as the volume remaining after exclusion of the region within the protein surface accessible to a probe of radius \( \sigma = 3 \) Å and the region outside the protein surface accessible to a probe of radius \( \sigma + \delta (\delta = 4 \) Å). The skin thus has a thickness of \( \delta \). The comparison was restricted to the skin within a sphere of 15-Å radius centered on the geometric center of FBP as positioned in the allosteric binding site of the crystal structure 1LDN.

To compare electrostatic potentials (\( \Phi \)), pairwise Hodgkin similarity indices (\( SI_{a,b} \)) were computed. For two proteins, a and b, \( SI_{a,b} \) is given by

\[
SI_{a,b} = 2\Sigma_{A} q_A \Phi_{A}/(\Sigma_{A}^{2} \Phi_{A}^2 + \Sigma_{B}^{2} \Phi_{B}^2) \tag{28}
\]

where the potentials are identical, \( SI_{a,b} = 1 \) if they are identical, \( SI_{a,b} = 0 \) if they are uncorrelated, and \( SI_{a,b} = -1 \) if they are anticorrelated. Corresponding pairwise electrostatic distances were defined as

\[
D_{a,b} = \sqrt{2 - 2SI_{a,b}}.
\]

**Computation of Energetically Favorable Binding Sites for Phosphate and Carboxylate Groups**—GRID software (29) was used to identify energetically favorable binding sites for P, and carboxylate (COO) probes in the catalytic and allosteric sites. The \( P_i \) probe was used to investigate binding of phosphate ions and FBP, whereas the COO probe was used for pyruvate. The binding of these probes in the catalytic site was assessed to examine the competition between \( P_i \) and pyruvate in the absence and presence of FBP and the consequent impact on the enzyme activity. Calculations were performed for the unliganded LDH models as well as for the LDHs with FBP positioned in the allosteric binding sites as in the template 1LDN structure. The computation of binding energies (\( E \)) was carried out at both pH 6 and pH 7.

**Algorithm to Computationally Estimate the Effect of \( P_i \)**—To estimate whether \( P_i \) has an activatory or an inhibitory effect on the enzymes, the computed probe binding energies were compared with those for the LDH from L. plantarum whose activity is known to be unaffected by \( P_i \). The binding energies of the \( P_i \) probe in the allosteric binding site (AS) and the COO probe in the catalytic binding site (CS) of LDH from L. plantarum were
defined as $E_{AS,\text{threshold}}$ and $E_{CS,\text{threshold}}$, respectively. For
the other LDH enzymes, the activity was considered to be
enhanced by $P_i$ if the binding energy of the $P_i$ probe in the
allosteric binding site ($E_{P_i}$) was $\geq E_{AS,\text{threshold}}$. When the bind-
ing energy of $P_i$ in the catalytic site ($E_{P_i}$) was $\geq E_{CS,\text{threshold}}$, no
inhibition by $P_i$ was anticipated. The magnitude of activation
and inhibition by $P_i$ was assessed by computing and analyzing
the following energy differences: $\Delta E_{AS} = E_{P_i} - E_{AS,\text{threshold}}$,
$\Delta E_{CS} = E_{P_i} - E_{CS,\text{threshold}}$, $\Delta E_{CS,pr} = E_{P_i} - E_{COO}$. A strong
enhancement of enzyme activity (designated by letter $A$) was
assigned when $\Delta E_{AS} \leq -2$ kcal/mol. The letter $a$ designates
weak activation, which was assigned when $-2 \leq \Delta E_{AS} \leq 0$
kcal/mol. Strong inhibition ($I$) was assigned when both $\Delta E_{CS} < 0$
and $\Delta E_{CS,pr} \leq -2$ kcal/mol. Weak inhibition ($i$) was assumed

**TABLE 2**
Effect of FBP on the activity of the LDH isoenzymes

Shown are the relative activities (%) compared with the values measured in the presence of 3 mM FBP. Assays conditions are as follows: 100 mM MES, 5 mM MgCl$_2$, 1 mM
NADH, 10 mM pyruvate at 30 °C. $K_{act}$ was measured as described under “Experimental Procedures.” See supplemental Tables S1–S3 for experimental measurements with absolute values. The error value for $K_{act}$ is defined as a percentage (%) of the measured value. n/a, not applicable as L. plantarum
LDH1 is not FBP-regulated; -, no measurements were performed.

| Organism, isoenzyme | Activity 3 mM FBP | Activity 0 mM FBP | $K_{act}$
|---------------------|------------------|------------------|----------|
|                     | pH 6 % | pH 7 % | pH 6 % | pH 7 % | pH 6 % | pH 7 % |
| *L. plantarum* LDH1 | 100 ± 5.6 | 100 ± 6.5 | 95.2 ± 7.7 | 103 ± 4.5 | n/a | n/a |
| *S. pyogenes* LDH | 100 ± 9.2 | 100 ± 8.0 | 1.2 ± 0.3 | 0.6 ± 0.0 | — | — |
| *E. faecalis* LDH1 | 100 ± 2.8 | 100 ± 8.7 | 12.7 ± 1.1 | 19.4 ± 0.5 | — | — |
| *L. lactis* LDH1 | 100 ± 0.2 | 100 ± 1.6 | 0.1 ± 0.05 | 3 ± 0.1 | 0.0003 ± 20% | 0.0005 ± 10% |
| *L. lactis* LDH2 | 100 ± 6.2 | 100 ± 2.6 | 22 ± 0.4 | 4 ± 1.3 | 0.0002 ± 15% | 0.140 ± 11.4% |
| *E. faecalis* LDH2 | 100 ± 1.6 | 100 ± 2.1 | 30.8 ± 0.7 | 37.3 ± 0.4 | 0.0001 ± 16.3% | 0.0001 ± 12.5% |

*a* Data from Ref. 14.

**FIGURE 2. The allosteric binding sites of the six LAB LDH enzymes.** The FBP binding site of each enzyme is shown in stick colored by atom type (upper) and
surface (lower) representations with the two (crystallographically determined for the LDH from *B. stearothermophilus*) binding positions of FBP shown as a
dot-contoured volume (upper) or in stick representation colored by atom type (lower). The electrostatic potential computed at pH 7 is mapped onto the
molecular surface of the enzymes and displayed from −7 (red) to +7 (blue) kcal/mol/e.
when both $\Delta E_{CS} < 0$ and $-2 \leq \Delta E'_{CS} \leq 0$ kcal/mol. For $\Delta E_{CS} \geq 0$, no competition between P$_i$ and COO in the catalytic site and thus no inhibition were expected ($\neg I$).

The following relations were used to deduce the overall effect of the presence of P$_i$ considering the possibility of P$_i$ binding at the allosteric site and the catalytic site: $A \land \neg I \rightarrow A; a \land \neg I \rightarrow a; \Box \land \neg I \rightarrow \neg E$ (no effect); $A \land I \rightarrow a \lor \neg E; \Box \land I \rightarrow I; A \land I \rightarrow \neg E \lor \Box \land I \rightarrow I$. Here, we use the common logical connectives (30) $\land$ for “and,” $\lor$ for “or,” $\neg$ for negation “not,” and $\Box$ for the cases when the allosteric binding site is occupied by FBP and thus not accessible for P$_i$.

**RESULTS AND DISCUSSION**

**Protein Expression and Purification**—We heterologously expressed the LDH1 isoenzymes of *L. plantarum* WCFS1, *S. pyogenes* M49 591, and *E. faecalis* V583 and the LDH2 isoenzyme of *E. faecalis* V583 in *E. coli* DH5α using the pASK-IBA2 vector and purified the enzymes by affinity chromatography. The calculated molecular masses of the isoenzymes carrying the Strep tag were 39.1 kDa for the LDH1 of *L. plantarum*, 37.2 kDa for the LDH of *S. pyogenes*, 37.4 kDa for LDH1, and 36.33 kDa for the LDH2 of *E. faecalis*. The success of the overexpression and purification of the isoenzymes was checked by SDS-PAGE (supplemental Fig. S2).

**Enzyme Kinetics**—The purified recombinant proteins were produced to study the kinetics of the isoenzymes and to determine the Michaelis constants ($K_m$) for NADH, pyruvate, NAD, and lactate at pH 6 and pH 7. Furthermore, the allosteric regulation of the isoenzymes by P$_i$ and FBP and the effect of ionic strength (NaCl) were investigated. $K_{act}$ values were measured when applicable.

As shown in Table 1, for each of the four LDH enzymes for which measurements were made, the $K_{act}$ values for pyruvate, NADH, lactate, and NAD were similar at pH 6 and at pH 7. Similar
results have been reported for the *L. lactis* LDH1 enzyme, whereas the *L. lactis* LDH2 was found to have higher affinity for its substrates at pH 6 than at pH 7 (14). As discussed in the next sections, the trends in activation/inhibition by P_i of each enzyme except *L. lactis* LDH2 were also similar at pH 6 and pH 7.

**Allosteric Mechanisms of LDH Activation**—From crystallographic studies, LDH was determined to mainly form a tetramer with two FBP binding sites and four active sites (31, 32). The axes along the FBP binding sites and the active sites were defined as *P* and *Q* axes, respectively (see Fig. 1). In the absence of FBP, two conserved positive residues, Arg and His, on the *P* axis interface can generate repulsion between subunits that in some bacterial organisms leads to a disassembly of the tetramer into dimers (16, 33).

Iwata *et al.* (15) determined the crystal structure of a 1:1 mixture of the inactive T-state and active R-state of *Bifidobacterium longum* LDH (Protein Data Bank code 1LTH). From this crystal structure, they proposed the following mechanism of LDH activation triggered by FBP binding. Addition of FBP neutralizes the repulsion of two dimers and stabilizes the tetrameric oligomerization of LDHs by hydrogen bond formation to Arg-173 localized on the *P* axis interface. A different rotameric state of Arg-173 in the complex with FBP then initiates a conformational change affecting the active site. Oxamate binding in the active site seems to complete the allosteric rearrangement from the inactive to the active state of the enzyme. It is accompanied by a helix (57–73 residues) sliding at the *Q* axis interface and subsequent replacement of His-68 on the next subunit by Arg-171 localized in the active site. The activation of the enzyme is thus mediated by the quaternary structural change.

Cameron *et al.* (16) proposed another mechanism of LDH activation. They found the LDH from *B. stearothermophilus* to be in an equilibrium of dimeric and tetrameric oligomers (Protein Data Bank codes 1LD8 and 1LDN) in which FBP binding promotes tetramerization. The crystallographic analysis revealed an allosteric mechanism that depended on intra-subunit conformational changes. In this model, upon FBP binding, Arg-173 rearranges such that a subsequent helix shift causes improved positioning of two adjacent charged residues, Arg-171 and Asp-168, in the active site of the same subunit, thereby affecting the substrate binding affinity.

Our LDH models are based on the tetrameric crystal structure of LDH from *B. stearothermophilus* (Protein Data Bank code 1LDN) with FBP, NAD^+, and oxamate bound, thereby imitating the activated R-form of the enzyme. Although the key residues that take part in the conformational rearrangement are conserved among the LAB LDHs (apart from *L. plantarum*), the allosteric mechanism by which conformational changes upon FBP or P_i binding are propagated can be different. A detailed analysis of the LDH dynamics would be necessary to investigate the LAB LDH allosteric mechanisms. However, this is not the aim of the present work, which is focused on the question of whether and how the binding of FBP and P_i in the allosteric and active sites (and therefore the regulation) varies among LDHs from a set of LABs that have adapted to different environments.

**Activation by FBP**—The LDH1 from *L. plantarum* has previously been described to be non-allosteric due to the presence of an aspartate residue in the effector binding site where the FBP-regulated LDHs have a histidine (17). Consistently, the activity of the LDH1 from *L. plantarum* was unaffected by the absence or presence of FBP at both pH values tested. The experimental results on activation by FBP are given in Table 2 and supplemental Table S1. In contrast to the *L. plantarum* LDH1, the activity of the *S. pyogenes* LDH decreased to 1.2% at pH 6 and 0.6% at pH 7 in the absence of FBP compared with its activity in the presence of 3 mM FBP. The effect of FBP on the activity of the *E. faecalis* LDH enzymes was less pronounced than that for *S. pyogenes*. In the absence of FBP, the *E. faecalis* LDH1 activity (relative to that in the presence of 3 mM FBP) decreased to 12.7% at pH 6 and 19.4% at pH 7, and that of LDH2 only decreased to 30.8 and 37.3% at pH 6 and pH 7, respectively. Thus, for activation by FBP, the *S. pyogenes* LDH resembles the LDH1 from *L. lactis* (14), the *E. faecalis* LDH2 resembles the LDH2 from *L. lactis*, and the *E. faecalis* LDH shows an intermediate activation level.

FBP is a negatively charged molecule, and therefore, to assess whether FBP activation could be related to the ability of the enzyme to bind FBP, we computed and compared the electrostatic potentials of the six LDH enzymes. Our findings reveal a distinct electrostatic potential for LDH1 from *L. plantarum* at its allosteric binding site (Fig. 2). In contrast to the other LDH enzymes, which display positive patches at the regions complementary to the negatively charged phosphate moieties of FBP, the allosteric binding site in LDH1 from *L. plantarum* is rather negatively charged. The negative electrostatic potential in the allosteric binding site might hinder or alter the mode of FBP binding. This supports the experimental evidence that LDH1 from *L. plantarum* in contrast to the other target LDHs studied is not FBP-regulated. By comparison of the electrostatic potentials in the vicinity of the FBP binding site using PIPSA (see “Experimental Procedures”), we observed the highest similarity...
between *L. lactis* LDH2 and *E. faecalis* LDH2 with an SI of 0.999, showing that the potentials are almost identical (Fig. 3). The *L. lactis* LDH1, *S. pyogenes* LDH, and *E. faecalis* LDH1 enzymes demonstrated high similarity with pairwise SI values over 0.94. As anticipated, the electrostatic potential of *L. plantarum* LDH1 was the most distinct, revealing a lower similarity to other enzymes, with pairwise SI values of about 0.8 (Fig. 3).

**Activation/Inactivation by Pi**—It has been noted previously (13, 14) that the presence of phosphate can affect the activation of *L. lactis* LDH. We hypothesized that Pi can affect LDH activity by binding at the allosteric site and/or at the catalytic site and that this binding can result in net activatory or inhibitory effects. We therefore tested experimentally and computationally the effects of Pi on the LDH activity in the absence and presence of FBP. Energetically favorable positions for the Pi probe in the allosteric site in the absence of FBP and in the catalytic binding site when FBP occupies the allosteric site are exemplified in Fig. 4. The experimental results are shown in Fig. 5, and the computational and experimental results are summarized and compared in Table 3. For experimentally measured absolute values, see supplemental Table S2. Here, we discuss each of the six LDH enzymes in turn in the order given in Fig. 5.
Regulation of Lactate Dehydrogenase Activity

As shown in Fig. 5A and consistent with the lack of FBP regulation, L. plantarum LDH1 activity is not significantly affected by the addition of Pi at concentrations up to 200 mM. However, the binding energies at which Pi and COO probes (the latter representing the carboxylate group of the pyruvate substrate) were computed to bind in the allosteric and catalytic binding sites, respectively, were taken as energy thresholds for deducing the effect of Pi on the activity of the other LDHs from the probe binding energy calculations (see “Algorithm to Computationally Estimate the Effect of Pi” under “Experimental Procedures”).

In contrast to L. plantarum LDH1, S. pyogenes LDH is weakly activated (Table 3) by Pi concentrations above 50 mM in the absence of FBP. The activity of the S. pyogenes LDH at pH 7 is about 5 times higher at 200 mM Pi, and at pH 6, 200 mM Pi leads to a 2.5-fold increase in activity (Fig. 5B). However, because of the intrinsically low activity of S. pyogenes LDH when effector is absent (0.8 and 0.6 μmol/min/mg of protein at pH 6 and pH 7, respectively), the activation by addition of Pi is considered to be weak. Computationally, at both pH values, we observed a favorable binding of the Pi probe at the allosteric binding site ($\Delta E_{\text{AS}} = -3$ and $-2$ kcal/mol) and no competition with a COO probe in the catalytic binding site ($\Delta E_{\text{CS}} = +1$ and 0 kcal/mol), thereby indicating activation by Pi. In the presence of FBP, no significant Pi-dependent changes ($\sim E$) in the activity of the S. pyogenes LDH were observed at either pH 6 or pH 7.

The activity of E. faecalis LDH1 is enhanced about 3-fold upon addition of 200 mM Pi in the absence of FBP (Fig. 5C). The computational results suggest activation of the E. faecalis LDH1 by Pi, although this is weaker than for S. pyogenes LDH due to less favorable binding of the Pi probe in its allosteric binding site ($\Delta E_{\text{AS}} = -1$ and 0 kcal/mol), thereby indicating activation by Pi. In the presence of FBP, the addition of Pi does not alter the activity of E. faecalis LDH1 (Fig. 5C), and this is also predicted from the computations (Table 3).

According to Gaspar et al. (14), the addition of 100 mM Pi in the absence of FBP at pH 6 enhances the activity of L. lactis.
Regulation of Lactate Dehydrogenase Activity

LDH1 by about 3-fold to 0.3% of the activity in the presence of 3 mM FBP. At pH 7, the activity is reduced by a factor of 4 to 0.7% of the activity in the presence of 3 mM FBP. Because the overall activity of the enzyme in the absence of FBP is very low, these variations with Pi concentration are not significant (Fig. 5D). Therefore, the Pi effect in Table 3 is designated as weakly activatory or insignificant (a ∨¬E) and weakly inhibitory or insignificant (i ∨¬E) at pH 6 and pH 7, respectively. Computationally, at pH 6, we observed a strong binding of the Pi probe in the allosteric binding site and a weak competition with the COO probe in the catalytic binding site, thereby resulting in a weak activation or no Pi effect. At pH 7, the lack of effect of Pi is more pronounced due to less favorable binding of the Pi probe in the allosteric binding site. In the presence of FBP, a weak inhibitory effect of Pi was observed both experimentally and computationally. The activity of the LDHs from L. lactis has been previously studied under different environmental conditions (13). The effect of varying the phosphate concentration on L. lactis LDH activity demonstrated a significant reduction of about 80% at 100 mM Pi, when 1 mM FBP was present (see Fig. 4 in Ref. 13). Gaspar et al. (14), however, obtained an inhibition of only 30% of L. lactis LDH1 activity at 100 mM Pi, in the presence of 3 mM FBP. According to Crow and Pritchard (Ref. 13, see Fig. 1B), at higher FBP concentration (10 mM), the phosphate inhibition is much less substantial. This observation may explain the weaker inhibitory effect for L. lactis LDH1 at 3 mM FBP than at 1 mM FBP.

According to Gaspar et al. (14), in the absence of FBP, the activity of L. lactis LDH2 was enhanced from 22 to 75% at pH 6, whereas at pH 7, the initially low activity of 4% remained the same after adding 100 mM Pi (Fig. 5E). This observation is consistent with the computational results. In the presence of FBP at pH 7, a weak inhibition (i) was experimentally measured as well as computationally assigned. However, at pH 6, no significant effect of Pi (¬E) was observed experimentally, whereas the computations suggest weak inhibition (i). We speculate that because the experiments were restricted to only 50 mM Pi, inhibition might indeed occur close to 100 mM as was measured by Gaspar et al. (14) for L. lactis LDH1 (pH 7).

Measurements for E. faecalis LDH2 in the absence of FBP activity showed an ~4-fold inhibitory effect (i) upon addition of Pi, at both pH 6 and pH 7. In the presence of FBP, a strong inhibition (¬i) of 6- and 8-fold for pH 6 and pH 7, respectively, was observed (Fig. 5F). The inhibitory effects were predicted computationally except for E. faecalis LDH2 at pH 6 in the absence of FBP for which a weak activation or no Pi effect instead of weak inhibition was expected.

P, Versus NaCl—To elucidate whether the observed activatory or inhibitory effect of Pi, in the absence of FBP was due to differences in ionic strength, we also measured enzyme activities with increasing NaCl concentrations for three LDHs at different pH values as control cases. The relative values are shown in Fig. 6, and the corresponding absolute values are presented in supplemental Table S3.

As can be seen in Fig. 6, NaCl did not influence the activity of S. pyogenes (pH 6) and E. faecalis (pH 7) LDHs. In contrast, for E. faecalis LDH2 (pH 6), addition of salts (Pi and NaCl) had an inhibitory effect (Fig. 6C). The inhibition upon addition of Pi presumably resulted from the stronger positive electrostatic potential of the enzyme at the phosphate binding positions (Fig. 2) and the high affinity of Pi to the catalytic and allosteric binding sites. Computation of Pi binding energies suggests more favorable binding to the catalytic site (−18 kcal/mol), thereby explaining a rapid inhibition at low Pi concentrations (up to 5 mM). At higher Pi concentration, binding to the allosteric binding site may also take place (−15 kcal/mol). This might have an activatory effect and thus suppress the strong inhibitory effect. The competition between inhibitory and activatory effects might lead to either a weak activation or no effect on enzyme activity upon Pi addition (Table 3). A different concentration dependence was observed upon addition of NaCl, suggesting a distinct mechanism. E. faecalis LDH2 probably demonstrates higher sensitivity to salt screening due to its stronger electro-
neut to explicitly account for the effects of P, on LDHs in kinetic models of LAB central metabolism. Moreover, the use of kinetic measurements and protein structure-based modeling provides a mechanistic understanding of the LAB LDHs, and this combined approach should be generally useful for studying other allosterically regulated enzymes.

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